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         Function and Gene Therapy

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13. ABSTRACT (Maximum 200 Words)

Two vestibular schwannoma cell lines, HEI-193 and HEI-182, as well as one vestibular nerve schwann cell line, HEI-286, were established using retroviral-mediated transfer of the HPV E6-E7 genes. HEI-193 cells, have a unique splice site mutation of the NF2 gene, while the NF2 gene in HEI-182 cells does not appear to be mutated. HEI-286 cells are expected to have the wildtype NF2 gene. Both immunocytochemistry and molecular biology techniques were used to demonstrate that these cell line are of Schwann cell origin. Comparison of the primary tumor with HEI-193 revealed the same NF2 mutation and an identical pattern of allele loss at multiple loci, indicating that the established cell line had maintained many of the properties of the original tumor. The immortalized cell lines were non-tumorigenic in severe combined immunodeficient (SCID) mice and/or nude mice, but did show altered growth properties such as higher proliferation rate and independence of Schwann cell growth factors. As part of our analysis, we also performed microarray analysis of gene expression in the cell lines as well as in tumors, including the original 193 tumor. The results obtained to date will help in our future studies aimed at elucidating the molecular pathogenesis of NF2.

14. SUBJECT TERMS
NF2, cell line, gene expression

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INTRODUCTION/PERSONNEL AND GRANT HISTORY:

The grant (DAMD 17-99-1-9491) was originally awarded to Dr. Gene Hung in 1999, but was transferred to Dr. Ali Andalibi in June 2002 due to the departure of Dr. Gene Hung from HEI (for Arena Pharmaceuticals) in March 2002. After Dr. Hung's departure, Dr. Andalibi assumed the mentorship of Dr. Juergen-Theodor Franzer, a post-doctoral scientist in the Section and the oversight of Dr. Huiqi Pan, the Research Associate in the Section. Dr. Pan left HEI in July 2002 to join Dr. Hung in his new position at Arena Pharmaceuticals. Dr. Fraenzer finished his postdoctoral term at HEI and returned to Germany in March 2003. Dr. Rodolfo Faudoa, a Post-doctoral Scientist had left the HEI in July 2001. Ms. Kathy Karouni joined the project as a Research Technician in January 2003, but left in January 2004 to commence her studies in pharmacy. Dr. Kathryn Rich joined the project in September 2003. A list of the staff members that have worked on projects related to this grant is given below:

Ali Andalibi, Ph.D., Scientist II and Director, New Technology and Project Development (June 2002 – Present)
David J. Lim M.D., EVP Research, Head, Department of Cell and Molecular Biology, Chief, Section on Pathogenesis of Ear Diseases
Gene Hung M.D., Scientist I and Chief, Section on Gene Based Therapy (Aug 1999 – March 2002)
Kathrine Rich, Ph.D. Visiting Scientist (Sept 2003 – Present)
Kathy Karouni, Research Technician (Jan 2003 – Feb 2004)
Juergen-Theodor Fraenzer, Ph.D., Post-doctoral Scientist (Dec 2000 - March 2003)
Huiqi Pan (Advanced Research Associate (June 2000-July 2002)
Rodolfo Faudoa M.D., Post-doctoral Scientist (July 1997 - July 2001)

PROJECTS:

The following projects have been proposed and approved for this grant:
1. Derivation of two schwannoma cell lines
2. Derivation of a Schwann cell line
3. Immunohistochemical characterization of schwannomas
4. Study of the molecular mechanisms by which NF2 regulates cell growth
5. Analysis of gene expression in cells and tumors

Of the above five projects the first four are complete. Publications describing the derivation of the first NF2 cell line, the role of NF2 in PDGFR degradation and immunohistochemical analysis

of schwannomas are attached. Other publications describing the establishment of schwannoma cultures, deletion analysis of NF2 DNA, as well as a method for detecting NF2 mutations are also attached. The final project entailing global gene expression analysis of cell lines and tumors is still on-going and the results will be available in the next few months.

PUBLICATIONS:

1: Fraenzer JT, Pan H, Minimo L Jr, Smith GM, Knauer D, Hung G. Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation. Int J Oncol. 2003 Dec;23(6):1493-500.

2: Hung G, Colton J, Fisher L, Oppenheimer M, Faudoa R, Slattery W, Linthicum F. Immunohistochemistry study of human vestibular nerve schwannoma differentiation. Glia. 2002 Jun;38(4):363-70.

3: Hung G, Li X, Faudoa R, Xeu Z, Kluwe L, Rhim JS, Slattery W, Lim D. Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2. Int J Oncol. 2002 Mar;20(3):475-82. PMID: 11836557 [PubMed - indexed for MEDLINE]

4: Bruder CE, Hirvela C, Tapia-Paez I, Fransson I, Segraves R, Hamilton G, Zhang XX, Evans DG, Wallace AJ, Baser ME, Zucman-Rossi J, Hergersberg M, Boltshauser E, Papi L, Rouleau GA, Poptodorov G, Jordanova A, Rask-Andersen H, Kluwe L, Mautner V, Sainio M, Hung G, Mathiesen T, Moller C, Pulst SM, Harder H, Heiberg A, Honda M, Niimura M, Sahlen S, Blennow E, Albertson DG, Pinkel D, Dumanski JP. High resolution deletion analysis of constitutional DNA from neurofibromatosis type 2 (NF2) patients using microarray-CGH. Hum Mol Genet. 2001 Feb 1;10(3):271-82.

5: Faudoa R, Xue Z, Lee F, Baser ME, Hung G. Detection of novel NF2 mutations by an RNA mismatch cleavage method. Hum Mutat. 2000;15(5):474-8.

6: Hung G, Faudoa R, Baser ME, Xue Z, Kluwe L, Slattery W, Brackman D, Lim D. Neurofibromatosis 2 phenotypes and germ-line NF2 mutations determined by an RNA mismatch method and loss of heterozygosity analysis in NF2 schwannomas. Cancer Genet Cytogenet. 2000 Apr 15;118(2):167-8.

7: Hung G, Faudoa R, Li X, Xeu Z, Brackmann DE, Hitselberg W, Saleh E, Lee F, Gutmann DH, Slattery W 3rd, Rhim JS, Lim D. Establishment of primary vestibular schwannoma cultures from neurofibromatosis type-2 patients. Int J Oncol. 1999 Mar;14(3):409-15.

BODY/RESEARCH SUMMARY

Key Research Accomplishments and Outcomes

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder of the nervous system that affects about 1 in 40,000 persons. NF2 is characterized by the presence of bilateral vestibular schwannomas, which are benign tumors of the vestibular portion of the eighth cranial nerve. NF2 patients can also suffer from meningiomas, cataracts and CNS tumors. Although bilateral vestibular schwannomas occur only in cases of NF2, unilateral vestibular schwannomas may occur sporadically and make up approximately 90% of the tumor cases. It is estimated that vestibular schwannomas account for approximately 8-10% of all intracranial tumors and nearly 80% of all cerebellopontine angle tumors (Nager 1985). The tumors cause pressure damage to neighboring nerves and in some cases, the damage to nearby vital structures, such as other cranial nerves and the brainstem, can be life-threatening. The most common symptoms are hearing loss, imbalance and tinnitus.

Mutations in the NF2 gene have been detected in approximately 70% of vestibular schwannomas and the gene product, merlin, is believed to act as tumor suppressor (Rouleau, Merel et al. 1993; Trofatter, MacCollin et al. 1993; Gutmann, Giordano et al. 1997). Mutation analyses of the NF2 gene from NF2 patients suggest that the inactivation of the NF2 gene and the consequent lack of a gene product, is the primary cause of this disease. Several studies have suggested that the NF2 gene protein directly interacts with plasma membrane molecules such as CD44 (Sainio, Zhao et al. 1997; Sherman, Jacoby et al. 1997), EBP50 (Reczek and Bretscher 1998) and hNHE-RF (Murthy, Gonzalez-Agosti et al. 1998), as well as with cytoskeletal proteins such as beta II-spectrin (Scoles, Huynh et al. 1998). These findings suggest that merlin may function to maintain normal cytoskeletal organization, and that like its homologues ezrin, radixin and moesin (ERM), it may work as a linker between the cell membrane and the cytoskeleton. As such, mutations of merlin may interfere with signaling cascades initiated at the cell surface. The mechanism, however, by which the NF2 protein mediates the tumor suppression is still unclear.

The establishment of *in vitro* models for the study of neurofibromatosis type 2 (NF-2) is essential to our efforts aimed at elucidating the role of the NF-2 gene product, merlin/schwannomin. Historically, the culturing of human Schwann or schwannoma cells has been problematic due to the difficulty of obtaining specimens, the small size of the specimens, the unavailability of schwann cell growth factors, and contamination by fibroblasts. The resources available at the House Ear Clinic, one of the major NF2 treatment centers in the US, however, have allowed us to overcome the obstacles listed above. As a result, not only have we developed a method for culturing primary schwannoma cells without fibroblast contamination, we have also established a frozen vestibular tumor bank, with over 500 frozen specimens and more than 70

primary culture stocks. In addition we have immortalized two vestibular schwannoma cell lines with distinct NF2 gene mutations, as well as a schwann cell line.

Specific Aims

Although there have been numerous important discoveries related to the biology of merlin and NF2, many questions about this disease still remain unanswered. The purpose of the studies proposed under this grant was to develop in vitro models for investigating the effects of the absence of neurofibromatosis-2 (NF2) gene expression in order to further our understanding of the biology of the NF2 gene product, Merlin.

The **Specific Aims** of the grant, were as follows:

Specific Aim 1: Establish a reproducible protocol for the primary culture of human Schwann and schwannoma cells and characterize NF2 gene function by studying cytoskeleton organization and tumor cell growth rate in culture.

Specific Aim 2: Immortalize one normal Schwann cell culture and two schwannoma cell cultures with different mutations and phenotypes and characterize the cell lines.

Towards Specific Aim 1, we have successfully made primary cultures of normal human schwann cells. This work has been previously published: Hung G, Faudoa R, Li X, Xeu Z, Brackmann DE, Hitselberg W, Saleh E, Lee F, Gutmann DH, Slattery W 3rd, Rhim JS, Lim D. Establishment of primary vestibular schwannoma cultures from neurofibromatosis type-2 patients. Int J Oncol. 1999 Mar;14(3):409-15.

Towards Specific Aim 2, we have derived two schwannoma cell lines and one Schwann cell line. The first schwannoma cell line has already been described (Hung G, Li X, Faudoa R, Xeu Z, Kluwe L, Rhim JS, Slattery W, Lim D. Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2. Int J Oncol. 2002 Mar;20(3):475-82). A description of the other two cell lines follows:

Materials and Methods:

Tissues and primary cultures: The first schwannoma cell line (HEI-193) that we derived was from a 58-year old male whose NF-2 related symptoms first occurred when he was 27. He had bilateral vestibular schwannomas, meningiomas, spinal tumors, and optic gliomas. As mentioned above, the results of this work was published in 2002. The second schwannoma cell line (HEI-182), on the other hand, was derived from the tumor of an eighteen-year old male who first became symptomatic at age 8. He was diagnosed with NF2 when he was 13 years old. In addition to bilateral vestibular schwannomas, this patient also had multiple spinal tumors and one meningioma. The Schwann cell line (HEI-286) was derived from a culture of vestibular nerve from

a 61 year-old male acoustic neuroma patient. After surgical removal, the tissues were placed in D-MEM/F12 medium and transported to the laboratory from the operating room. The tissues were then cut to 2 mm^3 in size and digested with 10 mg/ml collagenase/dispase (Boehringer Mannheim) in D-MEM/F12 medium (Gibco/Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2 for one hour. The digested material was washed and resuspended in D-MEM/F12 medium and plated. The cells were washed and transferred to new plates twice during the first 48 hours, and then plated in 100 mm laminin-coated dishes with modified D-MEM/F12 medium (Gibco/BRL) supplemented with insulin 10 mg/ml (Sigma), progesterone 3 X 10^{-8} M (Sigma), heregulin 10 nM (Genentech), bovine pituitary extract 3 mg/ml (Gibco/BRL), transferrin 10 mg/ml (Sigma), α-tocopherol 5 mg/ml (Sigma) and forskolin 5 mM (Sigma). The primary cells were grown under these serum-free conditions for two weeks.

Immortalization of primary cultures: The PA317 amphotropic packaging cell line was used for the immortalization procedure. These cells have been stably transfected with a replication-defective retrovirus construct, pLXSN16E6-E7, coding for the human papilloma virus type16 transforming oncoproteins E6 and E7 (9-13). PA317 cells were grown to 70-80% confluence, and supernatants were collected after 24 hours and stored in aliquots at –80°C. After two weeks of culture, the Schwann and schwannoma cells were trypsinized and seeded at a density of 5 x 10^5 in 60 mm laminin-coated plates containing D-MEM/F12 medium (Gibco/Invitorgen, Carlsbad, CA) supplemented with insulin 10 mg/ml (Sigma, St. Louis, MO), progesterone 3 X 10^{-8} M (Sigma, St. Louis, MO), heregulin 10 nM (courtesy of Genentech, South San Francisco, CA), bovine pituitary extract 3 mg/ml (Gibco/BRL), transferrin 10 mg/ml (Sigma), α-tocopherol 5 mg/ml (Sigma, St. Louis, MO) and forskolin 5 mM (Sigma, St. Louis, MO). Ten percent fetal bovine serum (FBS) (HyClone, Utah) was then added to the above medium. Twenty-four hours after the serum was added, 1 x 10^7 of retrovirus construct pLXSN containing human papilloma virus (HPV) E6-E7 genes and Neo^r gene were added to the culture medium (19, 20, 21). The cells were maintained at 37°C with 5% CO_2 and the medium was changed once per week. Once colonies were formed (about 5 weeks after the addition of the gene mixture), G418 (Gibco/BRL) was added to the final concentration of 0.4 mg/ml. After 8 – 10 days the G418 resistant colonies were cloned and expanded. These cells were recorded as Passage 0. The two schwannoma cell lines were designated HEI-193 and HEI-182, while the Schwann cell line was designated HEI-286.

Immunohistochemical characterization: The cultured cells were plated in laminin-coated chamber slides. Forty-eight hours later, plated cells were fixed in methanol/ethanol (1:1) at –20°C for 15 minutes. The slides were then washed three times with phosphate-buffered saline (PBS) and blocked with 10% normal goat serum (NGS) (Calbiochem, San Diego, CA) in PBS for 20 min at room temperature. Cells were incubated for one hour with primary antibody – S100 (1:500, Dako Corp., Carpenteria, CA) which is a marker specific for Schwann cells among peripheral nerve cell types. Expression of beta catenin, NCAM, myelin associated glycoprotein (MAG) (Santa Cruz Biotechnology, Santa Cruz, CA), NF2 (sc-331 and sc-332 (Santa Cruz Biotechnology, Santa Cruz, CA), and peripheral myelin protein 22 (PMP22) (sc-18535 (Santa

Cruz Biotechnology, Santa Cruz, CA), CNPase (2'-3'-cyclic neucleotide 3' phosphodiesterase) (Lab Vision, Fremont, CA) was also assessed. The cultured cells were also stained with mouse monoclonal anti-cytokeratin antibody C-11 (Sigma, St. Louis, MO) and monoclonal anti-vimentin antibody VIM-13.2 (Sigma, St. Louis, MO). After washing three times with PBS, secondary fluorescein-isothiocyanate (FITC) conjugated goat anti-rabbit antibody (1:15, Dako Corp.) was added and incubated for 30 min at room temperature. After three washes with PBS, the slides were covered with antifade/pipidien iodine or regular permount and viewed under a Zeiss fluorescent microscope (Zeiss, Germany).

Proliferation Assays: Proliferation of the HEI-193 cells was monitored by visualizing 5-bromo-2'deoxyuridine (BrdU) incorporation (22) using colorimetric immunoassay kit (Boehringer Mannheim), according to the manufacturer's recommendation. The proliferation rate of HEI-182 and HEI-286 cells was monitored by calculating the doubling time of the cells over a course of 5 days. Cells were seeded, in triplicate, at a density of 2×10^5 cells/6 cm dish. The first set of triplicate dishes were trypsinized and counted twenty-four hours later for each cell line. Another set of triplicate dishes were counted every 24 hours thereafter, for the next four days. The results were plotted and the population doubling time (DT) was calculated using the following formula: $Nt = N02^{tf}$, where Nt = number of cells at time t and $N0$ = number of cells initially, t= time (days) and f= requency of cell cycles per unit time (1/day).

Karyotype Analysis: Karyotyping was performed (Cell Culture Characterization Services, Orion Township, MI) when HEI-193 cell line was at Passage 17, HEI-182 cell line was at Passage 20 and HEI-286 cell line was at passage 46. In Brief, cells in logarithmic phase of growth were arrested with 0.06 mg/ml of colcemid for 1 hour, and then trypsinized and treated with 0.0375 M of KCl for 9 min. The cells were fixed with a mixture of methanol/acetic acid at a 3:1 ratio (23). Cell suspensions in the fixatives were dropped onto cold wet glass slides. The slides were air dried and stained with 4% Giemsa solution. Chromosomes were examined and counted to establish the ploidy distribution and constitutional aberrations. For trypsin Giemsa banding of chromosomes, the slides were heated at 60°C on a slide warmer for 18 hours, trypsinized for 3 seconds, stained with Giemsa stain for 11 min (24), washed, dried and then mounted in permount. The well-banded metaphases were karyotyped using AKSII image analysis system.

Loss of heterozygosity (LOH) analysis: For loss of heterozygosity analysis, genomic DNAs were extracted from the corresponding tumor tissue, blood and cultured HEI-193 cells by using TRIzol solution (Gibco/BRL). LOH analysis was performed using five selected microsatellite markers either flanking or within the NF2 gene: CRYB2 (centromeric) (25), D22S275 (centromeric) (MacCollin, unpublished), NF2CA3 (intragenic) (26), D22S268 (telomeric) (27) and D22S430 (telomeric) (28, 29). These five markers were amplified from tumor tissue, cultured cells or blood DNAs and analysed for their heterozygosities. One of each primer pair was fluorescently labeled with either one of three dye phosphoramidites – FAM, TET or HEX (ABI, Foster City). PCR amplifications and electrophoresis were performed as described

previously (30). The amplified markers were separated on an automated Genetic Analyzer ABI310.

Analysis of tumorigenicity in in mice: For HEI-193 cells, three of each SCID (Severe Combined Immunodeficient) mice and nude mice were used. 6×10^6 of HEI193 cells were subcutanously injected into right flank area of the mice. The mice were followed up to 8 weeks for signs of tumor growth. For HEI-182 and HEI-286 cells, 10 nude mice were used per line. The animals were injected subcutaneously with 1×10^6 cells and followed for 8 weeks for signs of tumor formation.

Tumors: Tumors were from 30 patients, 15 males and 15 females with confirmed NF2. Of the male subset, 6 were from individuals 16 years of age or under (young) and 9 from individuals over the age of 30 (old). Moreover, 4 of the tumors from the young group were small ($\leq$ 2cm) and two were large (2cm<). In the old group, three of the tumors were small ($\leq$ 2cm) and 6 were large (2cm<). Of the female subset, 6 tumors were from individuals 16 years of age or under (young) and 9 from individuals over the age of 30 (old). Furthermore, 4 of the tumors from the young group were small ($\leq$ 2cm) and two were large (2cm<). In the "old" group, 4 were small ($\leq$ 2cm) and five were large (2cm<).

RNA isolation: RNA was extracted from 13 NF2 tumors. Eight of the tumors were from males and five from females. Of the male subset, 4 were from individuals 16 years of age or under (young) and 4 from individuals over the age of 30 (old). Moreover, 2 of the tumors from each group were small ($\leq$ 2cm) and two were large (2cm<). Of the female subset, 3 tumors were from individuals 16 years of age or under (young) and 2 from individuals over the age of 30 (old). Furthermore, 2 of the tumors from the "young" group were small ($\leq$ 2cm) and one was large (2cm<). In the "old" group, one was small ($\leq$ 2cm) and one was large (2cm<). RNA was also extracted from HEI-193, HEI-182 and HEI-286 cells. Total RNA was extracted from quick-frozen tumors and the cell lines using TRIZOL according to the manufacturer's instructions (Invitrogen, Carlsbad CA). In addition, RNA was purified by the RNeasy cleanup system (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Oligonucleotide microarray: The detailed protocol for the sample preparation and microarray processing is available from Affymetrix at www.affymetrix.com (Santa Clara, CA). Briefly, 1 µg purified RNA was reverse transcribed by Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) using T7-(dT)24 primer containing a T7 RNA polymerase promoter. After synthesis of the second complementary DNA (cDNA) strand, this product was used in an in vitro transcription reaction to generate biotinylated complementary RNA (cRNA).

Fifteen micrograms of fragmented cRNA was hybridized to Human Genome U133 Plus 2.0 Array (Affymetrix) for 16 hours at 45°C with constant rotation at 60 rpm according to the Affymetrix protocol. The Human Genome U133 Plus 2.0 Array has complete coverage of the Human Genome U133 Set plus 6,500 additional genes for analysis of over 47,000 transcripts.

Each microarray was used to assay a single sample. After hybridization, the microarray was washed and stained on an Affymetrix fluidics station and scanned with an argon ion confocal laser, with a 488-nm emission and detection at 570 nm. The fluorescence intensity was measured for each microarray and normalized by global scaling to 2500 and to the average fluorescence intensity for the entire microarray. The data were imported into a Microsoft Excel database.

Data analysis: Data analysis was performed with the Genespring software version 7.1 (Silicon Genetics, San Carlos, CA). CEL files were imported into Genespring, and put through an RMA (robust multichip average) preprocessing probe level analysis. The normalizations that are performed in the RMA normalization steps ensure that the distribution of the expression values is comparable across the different chips or samples. Next the samples were normalized per chip and per gene to 50th percentile and to median, respectively. The cutoff was wet at 0.01 in raw signal measurement values. Then fold change was calculated for parameters of interest and the list of genes showing a 2-fold or higher level of change was generated and subsequently subjected to ANOVA using 1-way and 2-way test parametric tests not assuming equal variance. Genes with acceptable p values were then compiled and their function noted.

Results:

Derivation of Cell lines
We chose a retroviral mediated gene transfer technique to deliver the immortalization agent. The retrovirus technique has a relatively higher transduction efficiency in comparison to non-viral carriers and, most importantly, the retrovirus can reliably integrate into the target cell genome. Human papilloma virus type 16 E6 and E7 genes are considered oncogenes and have been shown to be associated with cervical cancers (Laimins, 1993). At the molecular level, the E6/E7 gene products interfere with the function of tumor-suppressor proteins p53 and Rb (Retinoblastoma). Thus, these gene products prevent cell cycle arrest without causing significant transformation (Band, et al., 1993; Helin, et al., 1992). Transduction of either the E6 or E7 gene alone can prolong a primary culture life through induction of hyperproliferation, but they have a very low frequency of transformation in primary cells. However, coexpression of both E6 and E7 genes can substantially increase the number of immortalization events (Hawley-Nelson, et al., 1989; Coursen, et al., 1997).

The HEI-182 cell line was derived from the tumor of an eighteen-year old male who was diagnosed with NF2 when he was 13 years old. The Schwann cell line (HEI-286) was derived from a culture of vestibular nerve from a 61 year-old male acoustic neuroma patient. The cells were immortalized with a retrovirus construct encoding HPV E6-E7 and Neo^r. After selection, the cells were characterized.

Morphology changes
Four to six weeks post retroviral transduction of the primary culture, colonies of short bipolar, spindle shaped cells began to emerge (data not shown). After approximately 10 passages

however, the cultured cells started to show morphological heterogeneity and a slowing in proliferation. The cultures reached crisis after about 15 passages. The surviving cells had a more compact appearance and have been morphologically stable since then.

Genotyping and Karyotyping of HEI-182 and HEI-193 tumors and Karyotyping of 286 cells:
After examination of 100 metaphases for each cell line – one from normal Schwann cells (HEI-NS286) and one from a schwannoma (HEI-NF182) - our results indicate that the HEI-286 cells are hypo-triploid male (XO) with most chromosome counts in the 60-68 range. Two X chromosomes were observed in each karyotype. There were no observed copies of chromosomes 8, 9, 13, and 14, one to three copies of chromosome 10, two copies of chromosomes 15, 16, 17, 18 and 20, two to three copies of chromosomes 1, 4, 5, 6, 7 and 12, and four copies of chromosome 3.

The results for HEI-NF182 cells suggest that these cells are also hypo-triploid human male (XO) and have chromosome counts in the 59-67 range. There were no observed copies of chromosomes 9, 13, and 14, one copy of chromosome 8, two copies of chromosomes 1,10, 15, 16, 17, 18, 19 and 21, two to three copies of chromosomes 20 and 22, three copies of chromosomes 6, 11 and 12, three to four copies of chromosomes 2, 4 and 5 and four copies of chromosomes 3 and 7. No germ line NF2 gene mutations in the coding region were found in either resected vestibular schwannoma or blood cells for HEI-182. Through the use of 5 microsatellite markers in the LOH analysis, the HEI-182 tumor cells did show loss of heterozygosity, but this loss was not seen in the blood cells. This result suggests that the germ line mutation was not in the coding region.

According to chromosome count, the HE-I193 cell line is hypodiploid human male (XO), with most chromosome counts in the 35 – 41 range. There were about 75% hypodiploid and 25% hypotetraploid metaphases observed. The composite karyotype was: 35-41, XO –(8, 9, 9, 13, 13, 14, 15, 17, 18, 19, 20 and 22) + [t (9, 13)(q10, q10) = M1, add (9)(q33) = M2, add (14)(q10) t(14;20)(q10,p10)=M3, t(15;?)(q10;?) = M4, der (19) t(13,19)(q12,q13) = M5] –(6, 10, 15, 17, 21) X15. There was loss of one complete copy of chromosome 8, 17, 18 and 22 in majority of metaphases.

Immunohistochemistry:
Cells were tested with a variety of antibodies in order to determine whether or not they had retained their original characteristics. As shown in Table I, neither HEI-182, nor the HEI-286 cells stained positive with an antibody to beta catenin, an epithelial cell marker. The cells were not positive for PMP22 and MAG, both markers for cells of the nervous system, nor for p75, another neuronal marker. HEI-182 cells were however, positive for S-100 while HEI-286 cells were positive for CNPase – both Schwann cell markers. Moreover, both cells were positive for NF2 A-19, an antibody that recognizes the amino terminus of merlin, suggesting that the molecule is expressed in HEI-286 cells, as expected, and that at least the amino terminus of merlin is expressed in HEI-182 cells. Neither cell type was positive for vimentin, suggesting that the

cells were not fibroblasts. The results for HEI-193 cells have already been published and it was shown that this cell line stained positively with S-100.

Antibody	HEI-182	HEI-286	HEI-193
Beta catenin	-	-	ND
S-100	+	-	+
Peripheral myelin protein 22 (PMP22)	-	-	-
NF2	+	+	ND
Nerve cell adhesion molecule (NCAM)	-	-	ND
Myelin-associated glycoprotein (MAG)	-	-	-
Vimentin	-	-	ND
Nerve growth factor receptor p75 (p75)	-	-	ND
2'-3'-cyclic neucleotide 3' phosphodiesterase (CNPase)	-	+	ND

Table I: Staining patterns of the cell lines using various markers.

Growth rate

The average doubling time of the HEI-182 cells was 36 hours compared to 41 hours for HEI-286 cells, over a 96 hour period (Table II). The data for HEI-193 cells is provided as a comparison and demonstrates that the two new cell lines are similar in their doubling behaviour to the previously reported HEI-193 cells. Moreover, after 96 hours, when the cells had reached confluence, the total number of cells was roughly the same in all three cultures, suggesting that the cells are of similar dimensions. It should be noted that no, patchiness nor piling up of cells, or other factors that could bias the cell count at confluence were observed.

Time (hours)	HEI-182	HEI-193	HEI-286
0	2.36×10^5	2.65×10^5	2.55×10^5
24	3.74×10^5	4.48×10^5	5.38×10^5
48	7.6×10^5	7.55×10^5	7.8×10^5
72	1.14×10^6	9.55×10^5	9.35×10^5
96	1.5×10^6	1.34×10^4	1.43×10^5
Doubling time	36 hrs	41 hrs	38 hrs

Table II: Cell counts at different time points.

Tumorigenicity in vivo

No sign of tumor growth was observed in nude mice (n=10) during the 8-week observation period post-inoculation of HEI-182 and HEI-286 cells.

Microarray Analysis

To date, we have analyzed 13 NF2 tumors from male and female subjects (Table III), as well as the three cell lines – HEI-193, HEI-286 and HEI-182. Eight of the tumors were from male patients – 4 from young subjects (16 years of age and younger) and 4 from old subjects (30 years of age and older). Within each group, two of the tumors were small ($\leq$ 2cm) and two were large (2cm<). Five of the tumors were from female subjects – 3 from young patients (16 years of age and younger) and two from old patients (30 years of age and older). Within the young group, 2 tumors were small ($\leq$ 2cm) and one large (2cm<). In the old group, one tumor was small ($\leq$ 2cm) and one large (2cm<). Comparisons of the gene expression profiles of the tumors were carried out and gene sets of transcripts with 2-fold or greater change in the analysis groups were derived. The gene sets were further analyzed using ANOVA with 1-way and 2-way test parametric tests not assuming equal variance.

Table III

Sex	Age	Tumor Size (cm)
Female	7	0.3
Female	9	1.2
Female	16	3.5
Female	38	3
Female	43	2
Male	12	1.5
Male	13	1.0
Male	15	5
Male	15	3
Male	31	1.5
Male	56	1
Male	50	4
Male	58	4

Age, sex and size of the tumors analyzed.

Genes differentially expressed in young and old females

Analysis of genes that were differentially expressed by a factor of 2 in tumors young versus old females identified 1481 genes. Statistically significant differences were then assessed using a parametric test, variances not assumed equal (Welch t-test), with p-value cutoff 0.01. This refined gene set contained 10 genes (Table IV). The genes were ranked according to their p-values and those annotated and with known function are described below.

Table IV

Affymetrix	Gene	p-value	Genbank Accession
202112_at	VWF	0.00067	NM_000552

221276_s_at	SYNCOILIN	0.002	NM_030786
242794_at	MAML3	0.00566	AI569476
204412_s_at	NEFH	0.00597	NM_021076
209392_at	ENPP2	0.00635	L35594.1
203815_at	GSTT1	0.00641	NM_000853
228391_at		0.00663	AI916528
230848_s_at	MGA	0.0075	BF438227
212875_s_at	C21orf25	0.00834	AP001745
236192_at		0.00896	BF447112

VWF (Von Willebrand factor)
VWF maps to 12p13. The VWF gene is approximately 178 kb long and contains 52 exons. The exons vary from 40 to 1379 bp in length, and the introns from 97 bp to approximately 19.9 kb. In a series of tissue extracts from 166 breast cancers, HGF (hepatocyte growth factor) content showed a strong positive correlation with von Willebrand factor content (Yao Y. et al., 1996). In a study of >200 invasive breast carcinomas, high IL-1 beta content in invasive carcinomas was significantly associated with higher contents of HGF, VWF, and TSP1 (Jin L. et al., 1997).

Syncoilin
Aparecida Nagai and colleagues demonstrated that (Aparecida Nagai et al 2003)
The transcripts of the Syncoilin gene were upregulated in tumor tissues overall as compared to the normal breast tissue, irrespective to the steroid hormone receptor status. Syncoilin gene encodes for a putative intermediate filament protein that interacts with desmin (Poon et al, 2002).

MAML3
Recently a mammalian homologue of the Mastermind gene of *Drosophila melanogaster*, *MAML1* (Mastermind-like-1 molecule) was cloned and determined that it functions as a transcriptional coactivator for Notch receptors. Two additional genes in this Mastermind-like gene family: MAML2 and MAML3 have also been characterized. The three *MAML* genes are widely expressed in adult tissues but exhibit distinct expression patterns in mouse early spinal cord development. All MAML proteins localize to nuclear bodies, share a conserved basic domain in their N termini that binds to the ankyrin repeat domain of Notch, and contain a transcriptional activation domain in their C termini (Wu et al. 2002).

Notch receptors initiate a highly conserved signaling pathway that influences cell fate decisions within multiple tissues and regulate the ability of precursor cells to respond to other developmental signals (Artavanis-Tsakonas et al., 1999). In mammals, Notch signaling has been shown to regulate neurogenesis (Beatus et al., 1998, Wang and Baress, 2000), as well as other aspects of organogenesis. In addition, Notch signaling is involved in other critical cellular processes such as proliferation and apoptosis (Miele et al. 1999). Consistent with the ability to

influence cellular differentiation in multiple tissues, mutations of Notch receptors and components of its signaling pathway have been associated with a number of diseases, including human T-cell leukemia (Notch1) (Ellisen et al., 1991), and breast carcinoma (Gallahan et al., 1996).

NEFH

The protein made by the NEFH (Neurofilament, heavy polypeptide 200kDa) gene is a component (subunit) of neurofilaments. Neurofilaments, which are assembled from light, medium, and heavy subunits, are essential for normal nerve function. They form a structural framework that helps to define the shape and size of nerve cells and to organize the contents of the cell. Cross-linking or bridging between neurofilaments maintains the width of nerve cells, which is important for the conduction of nerve impulses.
Bruder and colleagues (Bruder et al. 1999) described a patient who has a 7-Mb constitutional deletion between the NEFH and MB genes and who exhibits a severe NF2 phenotype (Bruder et al. 1999).

ECTONUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 2; ENPP2 (autotaxin)

Autotaxin (ATX/PDNP2), is a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells (Nam et al., 2000).

GSTT1

Glutathione S-transferase T1 (GSTT1) detoxifies some environmental carcinogens including cigarette smoke.   Geisler and colleages (Geisler et al., 2005) showed that certain alleles of GSTT1 were associated with developing squamous cell carcinoma.

MGA

Hurlin and coworker (Hurlin et al., 1999) identified a Max-interacting protein, Mga, which contained a Myc-like bHLHZip motif. Like Myc, Mad and Mnt proteins, Mga requires heterodimerization with Max for binding to the preferred Myc-Max-binding site CACGTG. In addition to the bHLHZip domain, Mga contains a second DNA-binding domain: the T-box or T-domain. The T-domain is a highly conserved DNA-binding motif originally defined in Brachyury and characteristic of the Tbx family of transcription factors. Mga binds the preferred Brachyury-binding sequence and represses transcription of reporter genes containing promoter-proximal Brachyury-binding sites. Surprisingly, Mga is converted to a transcription activator of both Myc-Max and Brachyury site-containing reporters in a Max-dependent manner. MAX gene associated is thought to function as part of a transcription factor complex.

Genes differentially expressed in small versus large tumors in young and old males
Analysis of genes that were differentially expressed by a factor of 2 in small and large tumors from young and old males identified 3344 and 2751 genes respectively. Statistically significant differences when grouped by tumor size, was then assessed using a parametric test, variances not assumed equal (Welch t-test), with p-value cutoff 0.05. This refined gene set contained 163

genes from the young group and 182 genes from the old group. The genes were ranked according to their p-values and the top 16 are described below (Table V and VI).

Table V

Affymetrix	Gene	p-value	Genbank Accesion
203290_at	HLA-DQA1	7.81E-06	NM_002122
212748_at	MKL1	0.000615	AB037859.1
209699_x_at	AKR1C2	0.000896	U05598.1
202747_s_at	ITM2A	0.00091	NM_004867
204151_x_at	AKR1C1	0.00104	NM_001353
237058_x_at	SLC6A13	0.00136	AI802118
223378_at	GLIS2	0.00191	AA705182
204048_s_at	KIAA0680	0.00197	NM_014721.1
231727_s_at	AD023	0.00201	NM_020679.1
201625_s_at	INSIG1	0.0021	BE300521
216836_s_at	ERBB2	0.00216	X03363.1

Genes differentially expressed in large and small tumors from young males

Table VI

Affymetrix	Gene	p-value	Genbank Accesion
203290_at	HLA-DQA1	7.81E-06	NM_002122
212551_at	CAP2	0.000437	NM_006366.1
227154_at	MGC15730	0.000466	AL566367
212748_at	MKL1	0.000615	AB037859.1
209699_x_at	AKR1C2	0.000896	U05598.1
202747_s_at	ITM2A	0.00091	NM_004867
204151_x_at	AKR1C1	0.00104	NM_001353
237058_x_at	SLC6A13	0.00136	AI802118
223378_at	GLIS2	0.00191	AA705182
204048_s_at	KIAA0680	0.00197	NM_014721.1
231727_s_at	AD023	0.00201	NM_020679.1
216836_s_at	ERBB2	0.00216	X03363.1

Genes differentially expressed in large and small tumors from old males

HLA-DQA1
Particular HLA polymorphisms result in well-defined associations with a large number of immunologically-mediated diseases, including some diseases with known dietary risk factors. For example, individuals of HLA-DQA1*0501, DQB1*0201 genotype have a greater than 200-fold increased risk of developing intolerance to dietary wheat gluten (coeliac disease) (Howell et al.,

2002). Moreover, radiation resistant types of B-cell chronic lymphoid leukemia (BCLL) show up-regulation of HLA-DQA1 expression (Vallat et al., 2003). An association between HLA-DQ1 and gastric cancer has also been shown (Garza-Gonzalez et al. 2004).

Megakaryoblastic leukemia (translocation) 1 MKL1
MKL 1 may play a role in chromatin organization, HOX differentiation pathways, or extracellular signaling and has been show to be involved in acute megakaryocytic leukemia (Mercher et al, 2001; Ma et al. 2001).

CAP2
Aenylate cyclase-associated protein, 2 interacts with the actin cytoskeleton and regulates cell polarity and has been shown to be involved in slowed DNA synthesis-induces filamentous differentiation in yeast (Kang and Jiang, 2005).

ITM2A
Integral membrane protein,type 2 A is involved in osteo and chondrogenic differentiation,involved in transcriptional regulation of cartilage or bone specific gene (Deleersnijder et al., 1996).

AKR1C2 and AKRC1
Human aldo-keto reductases (AKRs) of the AKR1C subfamily function in vitro as 3-keto-, 17-keto-, and 20-ketosteroid reductases or as 3alpha-, 17beta-, and 20alpha-hydroxysteroid oxidases. These AKRs can convert potent sex hormones (androgens, estrogens, and progestins) into their cognate inactive metabolites or vice versa. Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) AKR1C2 (3alpha-HSO3) is a progesterone metabolizing enzyme and was shown to be expressed in lower levels in tumorous as compared to normal breast tissue (Lewis et al, 2004).

SLC6A13
Solute carrier family 6 (neurotransmitter transporter, GABA), member 13 is a GABA transporter expressed in many tissues types but mainly the CNS and kidney.

GLIS2
Kruppel-like zinc finger protein GLIS2 is a relatively proline-rich, basic 55.8-kDa protein. Its five tandem Cys(2)-His(2) zinc finger motifs exhibit the highest homology to those of members of the Gli and Zic subfamilies of Kruppel-like proteins. Glis2 behaves as a bifunctional transcriptional regulator and both its activation and repressor functions may play an important role in the regulation of gene expression during embryonic development, especially in kidney development and neurogenesis (Zhang et al., 2002)

PHACTR2 (KIAA0680)
PHACTR2 is a phosphatase and actin regulator (Allen et al., 2004).

AD023 protein
Is expressed in a variety of tissues although its expression is high in T-cell leukemia and glioblastoma according to Unigene & EST expression information (http://harvester.embl.de/harvester/Q8N4/Q8N4Q5.htm) (Strausberg et al., 2002).

ERBB2
Avian erythroblastic leukemia viral (v-erb-b2) oncogene homolog 2 (LL) encodes a polypeptide with a kinase domain that is highly homologous with, but distinct from, that of the epidermal growth factor (EGF) receptor. Overexpression of ERBB2 has been implicated in the neoplastic transformation of prostate cancer. Overexpression of ERBB2 confers Taxol resistance in breast cancers. Amplification of the gene encoding the ERBB2 receptor tyrosine kinase is critical for the progression of several forms of breast cancer. In a large-scale clinical trial, treatment with Herceptin (trastuzumab), a humanized blocking antibody against ERBB2, led to marked improvement in survival (Slamon et al 2001).

<u>Genes differentially expressed between HEI-193, HEI-182 NF2 cell lines and HEI-286 Schwann cell line</u>
Analysis of genes that were differentially expressed by a factor of 5 between HEI-286 cells and HEI-182 cells and a factor of 3 between HEI-286 cells HEI-193 cells identified 365 and 121 genes, respectively. Statistically significant differences were then assessed using a parametric test, variances not assumed equal (Welch t-test), with p-value cutoff 0.01. This refined gene set contained 27 genes that were expressed differentially between HEI-286 and HEI-182 cells and 11 genes that were expressed differentially between HEI-286 and HEI-182 cells. Assessment of The annotated genes were ranked according to their p-values and are listed in below (Tables VII and VIII).

Table VII

Affymetrix	Gene	p-value	Genebank Accesion
205110_s_at	FGF13	0.00171	NM_004114
203789_s_at	SEMA3C	0.00409	NM_006379
211006_s_at	KCNB1	0.00433	L02840.1
212298_at	NRP1	0.00498	BE620457
205000_at	DBY	0.00557	NM_004660
219279_at	FLJ20220	0.00558	NM_017718
223058_at	MGC11034	0.00561	AL136885.1
225325_at	FLJ20160	0.00656	AA133311
227058_at	FLJ14834	0.00667	AW084730
201912_s_at	GSPT1	0.00677	NM_002094
222421_at	UBE2H	0.00708	AW205983
218613_at	DKFZp761K1423	0.00751	NM_018422

202695_s_at	STK17A	0.00751	NM_004760
202121_s_at	BC-2	0.00814	NM_014453
212590_at	RRAS2	0.00818	BG168858
207303_at	PDE1C	0.00831	NM_005020
201852_x_at	COL3A1	0.00841	AI813758
221702_s_at	BLP2	0.00848	AF353992.1
229500_at	C4orfl	0.00853	AI609256
225579_at	MGC33602	0.00914	AK001091.1
203474_at	IQGAP2	0.00965	NM_006633

Genes differentially expressed by a factor of 5 between HEI-286 and HEI-182 cells

Table VIII

Affymetrix	Gene	p-value	Genebank Accesion
203789_s_at	SEMA3C	0.00409	NM_006379
212298_at	NRP1	0.00498	BE620457
205000_at	DBY	0.00557	NM_004660
243489_at	ROCK1	0.00664	BF514098
202121_s_at	BC-2	0.00814	NM_014453
212590_at	RRAS2	0.00818	BG168858
224894_at	YAP1	0.00881	BF210049
225579_at	MGC33602	0.00914	AK001091.1

Genes differentially expressed by a factor of 3 between HEI-286 and HEI-193 cells

Conclusions

Using retroviral mediated gene transfer, we have established an NF2 cell line (HEI-182) and a Schwann cell line (HEI-186). Another NF2 cell line, HEI-193, was developed earlier and has been reported. The clinical manifestations of the NF2 patient were severe, with an early age of onset and numerous tumors. A retroviral vector carrying the human papilloma virus type 16 (HPV16) E6 and E7 genes was used for the immortalization because of its high transduction efficiency and its ability to stably integrate into the target cell genome. E6 and E7 are transforming proteins that have been found associated directly with cervical cancer (33). The E6/E7 gene products interfere with the function of tumor-suppressor protein p53 and Rb (Retinoblastoma), respectively, thereby preventing cell cycle arrest (34, 35). Co-expression of E6 and E7 genes, can substantially increase the number of immortalization events (36,37).

The Schwann cell origin of the immortalized cells was confirmed by staining with CNPase and S100. The cells are non tumorigenic and express a protein that is recognized by an antibody to

the amino terminus of merlin, thus suggesting that the mutation results in the expression of an altered protein. The nature of the alteration is yet to be explored.

In conclusion, we have derived a second human schwannoma cell line, as well as a Schwann cell line by immortalization with a retrovirus construct containing human papilloma virus E6/E7 genes. This immortalized schwannoma cell lines, HEI-182 and HEI-286 grow well, have a stable morphology, stain positively for Schwann cell markers and are not tumorigenic in nude mice. The cell lines should prove to be useful for further molecular characterization of NF2 gene function. In addition, they may be potentially useful as an *in vitro* model for testing therapeutic approaches.

The results of our analysis of differential gene expression in large and small tumors from young versus old patients suggest that a set of genes, including many associated with cancers may serve as good markers for each tumor type. The results obtained will next be confirmed using quantitative PCR and the studies will be continued to include more samples. Moreover, analysis of differential gene expression between two cell lines derived from NF2 tumors and one from Schwann cells indicates that the NF2 lines do show some similarities and differ from the Schwann cell line with similar patterns of differential expression for a number of genes.

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Appendices
See attached manuscripts

Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation

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Abstract. The loss of NF2 gene function leads to vestibular nerve schwannoma formation in humans. The NF2 gene product, Merlin/Schwannomin, has recently been found to interact with the two PDZ domains containing protein EBP50/NHE-RF, which is itself known to interact with the PDGF receptor (PDGFR) in several cell types. In this study, an up-regulation of both PDGFR and EBP50/NHE-RF, and an interaction of both proteins were found in primary human schwannoma tissue. Furthermore, using an adenoviral vector mediated gene transfer technique, changes in the phenotypic characteristics after NF2 gene restoration in a newly established NF2 gene-mutated human schwannoma cell line (HEI 193) were investigated. The overexpression of Merlin/Schwannomin in HEI 193 led to an inhibition of cell proliferation under serum-free conditions. Upon PDGF stimulation in culture, Merlin/Schwannomin appeared to inhibit the activation of the MAPK and PI3K signaling pathways, impinging on the phosphorylation of Erk 1/2 and Akt, respectively. The data also show that PDGFR is more rapidly internalized by the schwannoma cells overexpressing NF2. Therefore, this process is suggested as a model for a mechanism of Merlin/Schwannomin tumor suppressor function, which intermediates acceleration of the cell surface growth factor degradation.

Introduction

Bilateral vestibular nerve schwannomas are the hallmark of neurofibromatosis type 2 (NF2), a rare genetic disorder with an incidence rate of approximately 1:37000 live births (1). Patients with NF2 develop neoplastic and malformative lesions of Schwann cells, specifically schwannoma and

schwannosis next to others (meningiomas, ependymomas, astrocytomas and gliomas; 2,3). These tumors are thought to be caused by loss of functions for the gene product of NF2, Merlin/Schwannomin, a 595 amino acid protein, which acts as a tumor suppressor (4,5). In most schwannoma tissues, a loss of heterozygosity in addition to the germ line NF2 mutation, can be detected (6). Furthermore, homozygous NF2^{-/-} mice show characteristics similar to human patients suffering from NF2 (7).

The 65-70 kDa sized Merlin/Schwannomin belongs to the band 4.1 protein super-family (8). Its closest members, ezrin, radixin and moesin (ERM), are cytoskeleton-associated proteins serving as structural linkers between the cytoskeleton and the plasma membrane (9). However, there are differences in the C-terminal halves of these proteins: in Merlin/Schwannomin the actin-binding domain is missing. Merlin/Schwannomin shares 35% homology with the ERMs in this region, compared to 64% with the N-terminus. Thus, it will be assumed that Merlin/Schwannomin may function in a manner distinct to the ERMs (10). This hypothesis is supported by the fact that so far only one ERM protein and Merlin in *Drosophila* has been found (D-Moesin-like, D-Merlin; 11). There is, however, biochemical evidence of a major cytoskeletal attachment domain at the N-terminus of Merlin/Schwannomin accompanied by regulatory sequences in the C-terminal half (12).

The N-terminus of Merlin/Schwannomin has also been reported to interact with EBP50/NHE-RF (ERM-binding phosphoprotein 50/Na^{+}-H^{+} exchanger regulatory factor; 13,14), a 357 amino acid-long membrane associated protein which contains two PDZ domains. EBP50/NHE-RF was originally identified by its mediating protein kinase A regulation of the renal brush-border Na^{+}-H^{+} exchanger (15). The first PDZ domain of EBP50/NHE-RF recognizes the D-S/T-x-L motif at the carboxyl terminal of several cell surface receptors such as β_2-adrenergic receptor, CFTR, purinergic P2Y1 receptor and platelet-derived growth factor (PDGF) receptor (16). Recent findings indicate this protein to be involved in β_2-adrenergic receptor recycling on the cell surface (17).

The ligand PDGF acts as a major connective tissue cell mitogen, as well as a chemoattractant. In combination with its receptor, it is involved in development of different cancers (18,19). In NF1-mutated neurofibrosarcomas, upregulation of

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PDGFR was found (20). The activation of PDGFR through its ligand leads to activation of the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK; also named extracellular-signal regulated kinases, Erk 1/2) signaling pathways. Activation of these pathways stimulates survival, growth, and proliferation of neuronal cells (21). Signaling through the PI3K pathway can lead to the activation of the serine/threonine-kinase Akt (also named protein kinase B) to inactivate several components of the apoptotic machinery resulting in increased cell survival (22,23). In contrast, the Erks act on transcription factors in the nucleus and influence cell cycle progression (24).

To gain more insight into NF2 gene function, its interactions with EBP50/NHE-RF and PDGFR and its influence on signaling pathways, we examined the expression levels of EBP50/NHE-RF and PDGFR, and characterized the interaction of these two proteins in primary human vestibular schwannoma tissues. By using a human immortalized schwannoma cell line (HEI 193) from a NF2-patient and an adenovirus carrying the NF2 gene for targeted overexpression, we furthermore demonstrate that Merlin/Schwannomin mediates/causes a cell growth inhibitory effect perhaps through the acceleration of cell surface growth factor receptors degradation.

Materials and methods

HEI 193 cells. The establishment of this human schwannoma cell line was described previously (25). A retrovirus-mediated gene transfer technique was applied for that study. A retrovirus carrying the E6- and E7-genes of the human papilloma virus was generated, and used to transduce primary schwannoma cell cultures. One primary schwannoma culture from a NF2-patient (HEI 193, 58-year old male), was successfully immortalized. His phenotype was characterized by bilateral vestibular schwannomas, meningiomas, spinal tumors, and optic gliomas. A splice site germ line mutation in the NF2 gene was found in the HEI 193 cell line, as well as in the vestibular schwannoma tissue and blood. This cell line was near diploid with a loss of one copy of chromosome 22 by cytogenetic analysis. The cell line was maintained at 37°C in F12/DMEM (Irvine Scientific) adding 10% fetal bovine serum (FBS, Hyclone).

Human vestibular schwannoma tumors. Fresh human vestibular tumor tissue was removed from NF2-patients, immediately placed in liquid nitrogen, transported to the laboratory, and stored at -80°C.

Antibodies. Monoclonal antiserum for phosphorylated Erk 1/2 and polyclonal antibody for phosphorylated Akt were purchased from Cell Signaling Inc. Polyclonal antibodies (A-19 and C-18) for Merlin/Schwannomin were purchased from Santa Cruz Biotechnology Inc., CA. Polyclonal antiserum for EBP-50 was kindly provided by Dr David Reczek at Cornell University. Polyclonal antiserum for PDGFR was purchased from R&D Systems, Inc.

Adenoviruses. Replication-defective recombinant adenoviruses were constructed as described previously (26). The coding regions E1-E17 of the NF2 gene (isoform II, 590 amino acids) were inserted into a modified pXC vector containing the Rous sarcoma virus (RSV) long terminal repeat and the bovine growth hormone polyadenylation tail. A second expression corset containing RSV promoter driving green fluorescent protein (GFP) and bovine growth hormone polyadenylation was placed downstream of the NF2 expression corset. Recombinant adenoviruses were generated, plaque-purified, and isolated on a cesium chloride gradient as described by Romero and Smith (26). The ratio of viral particles to plaque-forming unit (pfu) for these viruses ranged from 123:1 to 260:1.

Transduction of HEI 193 cells. All transductions were performed in confluent cell amounts in 100 mm petri dishes and 500 MOI. Cells were freshly split one day prior to transduction. After 40-h incubation the media including abundant viruses were washed off. The cells were plated 24 h later into 24 wells arrays in a final volume of 1 ml, and again 24 h later the medium was changed including one wash with PBS, either to a serum-free condition or to a new 10% FBS-containing one. Two to four days after onset of starvation the degradation experiments were performed.

In vitro binding assays. A cell density of 5×10^6 cells were defined in F12/DMEM medium supplemented with 10% FBS and plated in 100 mm dishes 24 h prior to adenovirus transduction. FBS was removed from the culture medium 40 h post-transduction. Cells grown under serum starvation condition for 48 h, were treated with F12/DMEM containing 40 ng/ml PDGF-BB (R&D System) for 5 min (10 and 30 min initially, respectively), then lysed in cell lysis buffer (Cell Signaling).

In vitro PDGFR degradation assay. All experiments involving $125I^*$ were performed at UCI in the laboratory of D. Knauer. $125I^*$-Na was obtained from Amersham Corp.. PDGF was labeled using the Iodogen method (27). Specific activities for $125I^*$-PDGF ranged from 3000 to 4000 cpm/ng of protein.

The starved cells were washed once with 1 ml binding medium (Dulbecco's modified Eagle's medium without serum, containing 1 mg/ml bovine serum albumin (BSA) and 20 mM HEPES buffer, pH 7.4), and then 300 μl of binding buffer containing 40 ng/ml $125I^*$-PDGF was added and incubated for a period of up to 4 h at 37°C. From the media 100 μl aliquots were taken at the indicated times and added to microfuge tubes on ice containing 1 ml 12% trichloroacetic acid (TCA) and 50 μl 3% BSA (in 20 mM Tris-HCl, 150 mM NaCl). After 1 h incubation on ice, precipitates were pelleted by centrifugation (10000 x g, 10 min), and the supernatants containing nonprecipitable radioactivity were counted in a γ-counter (Beckman). Background degradation determined from complexes incubated in medium in the absence of cells was subtracted from each time point.

Western blotting. The transduced cells were lysed in cell lysis buffer (Cell Signaling). The concentration of protein extracts dissolved in 1% SDS was measured with the BCA assay kit (Pierce). Total protein (25 μg) was separated by 8% SDS-PAGE and transferred to Hybond ECL nitrocellulose

membrane (Amersham) in Towbin buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). The membrane was blocked with 7% milkpowder in 0.1% Tween-20 in PBS (PBS-T) and washed with PBS-T. Primary antibodies (Erk 1/2 1:2000, Akt 1:1000) were incubated with the membrane for 1 h and then washed with PBS-T. The membrane was then incubated with goat anti-rabbit antibody coupled with horseradish peroxidase for 45 min and subsequently washed by PBS-T. The presence of the secondary antibody bound to the membrane was detected by enhanced chemiluminescence (ECL, Amersham).

Co-immunoprecipitation. Total of 250 mg of frozen tumor tissue was homogenized and lysed in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA) on ice. Different amounts of lysis super-natants were immunoprecipitated by antibody against PDGFR (1 μg/ml) in lysis buffer with 35 μl of protein A/G beads (Amersham Pharmacia, Sweden) at 4°C overnight. The beads were washed with lysis buffer three times and resuspended in loading buffer and boiled for 5 min. The supernatants were loaded onto 8% SDS-PAGE gels. The proteins were transferred onto Hybond ECL paper and blotted with EBP50 antibody in blocking buffer (7% milkpowder, 0.1% Tween-20 in PBS). The membrane was then incubated with goat anti-rabbit antibody coupled with horseradish peroxidase for 45 min and subsequently washed by PBS-T. The presence of the secondary antibody bound to the membrane was detected by enhanced chemiluminescence.

Results

Interaction and overexpression of PDGFR and EBP50/NHE-RF in schwannoma cells. To identify whether platelet-derived growth factor (PDGF) is involved in schwannoma tumorigenesis, PDGF receptor (PDGFR) expression in schwannoma tissues was examined by Western blot analysis. Recently, EBP50/NHE-RF has been found able to potentiate PDGFR autophosporylation and Erk activation (16). Compared to normal nerve tissue, a significantly higher expression of PDGFR, EBP50/NHE-RF and phosphorylated Erk 1/2 were found in all schwannoma tissues examined (Fig. 1A and B). It is intriguing that two bands (48 and 50 kDa) of EBP50/NHE-RF could be seen in some tumors (Fig. 1A, T8; less striking to see in T3, T4 and T7). Similar doublets were originally described in stomach, lung and spleen, where the two bands corresponded to 50 and 52 kDa, respectively (13). The higher molecular weight band is thought to be the phosphorylated form. To determine if PDGFR and EBP50/NHE-RF interact in schwannoma, a co-immunoprecipitation assay was performed. Immunoblots of EBP50/NHE-RF after immunoprecipitation of PDGFR demonstrates the *in vivo* interaction between both proteins in a linear manner of concentration (Fig. 1C).

Inhibition of schwannoma cell proliferation by overexpression of NF2 in serum-free conditions. To see whether the restoration of NF2 gene function effects the proliferation in the NF2-mutated cells, an adenovirus vector with dual expression cassettes encoding the wild-type NF2 cDNA (isoform II) and
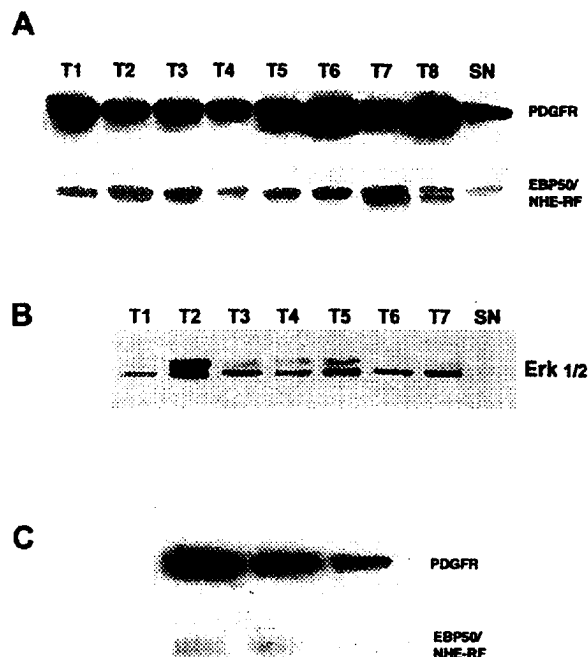

Figure 1. *In vivo* overexpression of PDGFR and EBP50/NHE-RF in NF2 tumor tissue, demonstration of interaction. A, Tumor tissues from eight different NF2-patients were tested for PDGFR and EBP50/NHE-RF expression compared to expression in the sciatic nerve of a non-NF2 corpse by Western blot analysis. B, Seven different tumor tissues were shown to express a high amount of Erk 1/2, while these proteins are almost undetectable in the sciatic nerve. C, Interaction of PDGFR and EBP-50/NHE-RF was demonstrated by Co-IP in three different protein concentrations being linearly correlated.

green fluorescent protein (GFP) cDNA, and a control vector expressing only the GFP gene were constructed. Both vectors were able to efficiently transduce the NF2 gene mutated HEI 193 schwannoma cells (Fig. 2). All cultures were plated at a density of 10^5 cells in 1 ml medium per well 64 h after the transduction. 24 h after replating, the serum containing medium was removed and one half of the wells were kept on serum-free conditions. The proliferation rates of cells grown with or without serum were determined by cell counting using a hexacytometer. Under serum-free condition, cells which overexpressed Merlin/Schwannomin grow significantly slower than the cells expressing GFP alone. Interestingly, this result was reversed when cells were grown in 10% serum-containing medium, the NF2 overexpressing cells grew faster than the cells expressing only GFP (Fig. 2). Evidently, there was seen no detectable difference for Merlin/Schwannomin expression in HEI 193 cells grown either with or without serum as the constant high green fluorescence indicates (Fig. 2). Moreover, cells without adenovirus transduction grew at the fastest rate, indicating a potential cytotoxicity effect or interference of cell cycling induced by adenovirus transfection and/or GFP-overexpression.

Overexpression of the NF2 gene leads to down-regulation of PDGF mediated MAP kinase and PI3 kinase activation. Indeed, PDGF was previously shown to activate the Akt
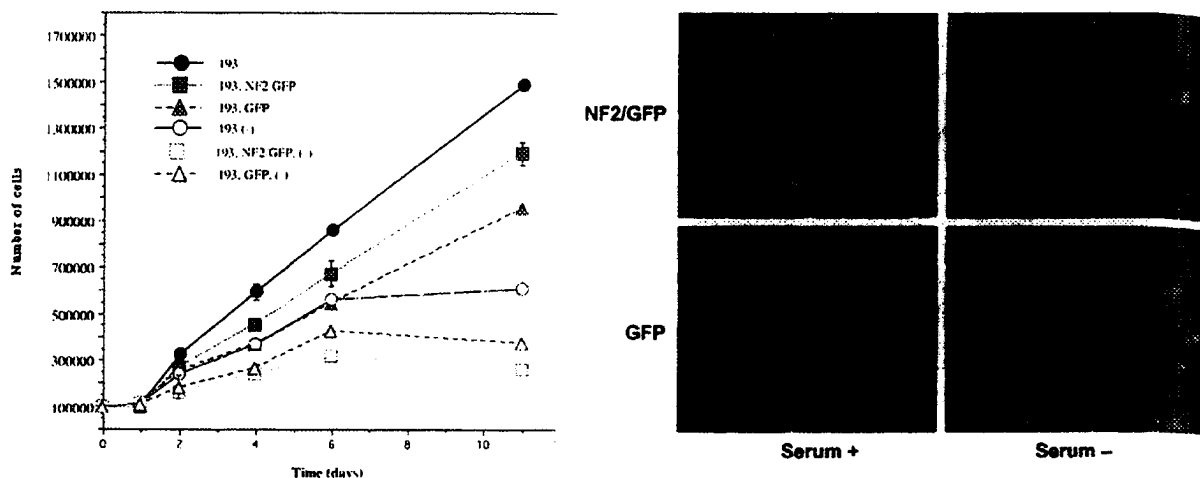
Figure 2. Cell proliferation of HEI 193, HEI 193 overexpressing NF2/GFP, HEI 193 overexpressing GFP. In medium containing 10% serum, the NF2 overexpressing cells proliferate faster than only GFP transduced ones. Under serum-free conditions the growth speed converts. Under both conditions, untransduced schwannoma HEI 193 cells grow at the most rapid rate. On the right is shown in all four investigated conditions the same level of fluorescence (HEI 193 cells showed no fluorescence).

pathway in primary mouse embryo fibroblasts, NIH 3T3 cells and Rat-1 cells in a rapid and specific manner (22). In order to demonstrate that PDGF is able to activate both MAP kinase and PI3 kinase in NF2 gene mutated schwannoma cells, the phosphorylated Erk 1/2 and Akt were measured upon PDGF stimulation in the HEI 193 cells. Western blot analysis was used to examine phosphorylated protein levels for Erk 1/2 and Akt from cells treated with 40 ng/ml PDGF at indicated time-periods prior to cell lysis. In NF2-mutated cells Erk 1/2 (42/44 kDa) and Akt (66 kDa) phosphorylation was apparent 5 min after addition of PDGF at the highest level (Fig. 3A). To examine if restoration of normal NF2 gene function in the schwannoma cells would influence PDGF-induced signal pathways, the phorsphorylation status of these kinases were compared in HEI 193 cells overexpressing either NF2/GFP or GFP alone. The results obtained from these experiments indicate that overexpression of Merlin/Schwannomin inhibits activation of Erk 1/2 and Akt in HEI 193 cells (Fig. 3B). To see whether the anti-proliferative effect of wild-type NF2 is mediated through PDGFR-EBP50/NHE-RF-Merlin/Schwannomin interaction, another Schwann cell mitogen, heregulin, was tested. Heregulin transduces signals by binding to either the Her-3 and Her-4 receptors complexes. Signaling of this pathway is similar to that of the PDGFR pathway, but independent of the here described interaction EBP50/NHE-RF-Merlin/Schwannomin. In the presence of heregulin a similar reduction in the phosphorylation levels of Erk 1/2 and Akt was apparent (Fig. 3C), indicating that NF2's growth inhibitory role is not mediated through PDGFR-EBP50/NHE-RF.

Interestingly, after the activation of PDGFR through PDGF treatment in Merlin/Schwannomin overexpressing cells, the levels of phosphorylated Akt were lower than those levels in untreated cells. Identical results were repeatedly seen in experiments with longer starvation of up to 6 days (data not shown). In these cases, the total amount of Akt was unchanged, as proved by using an antibody versus non-phosphorylated Akt (data not shown).
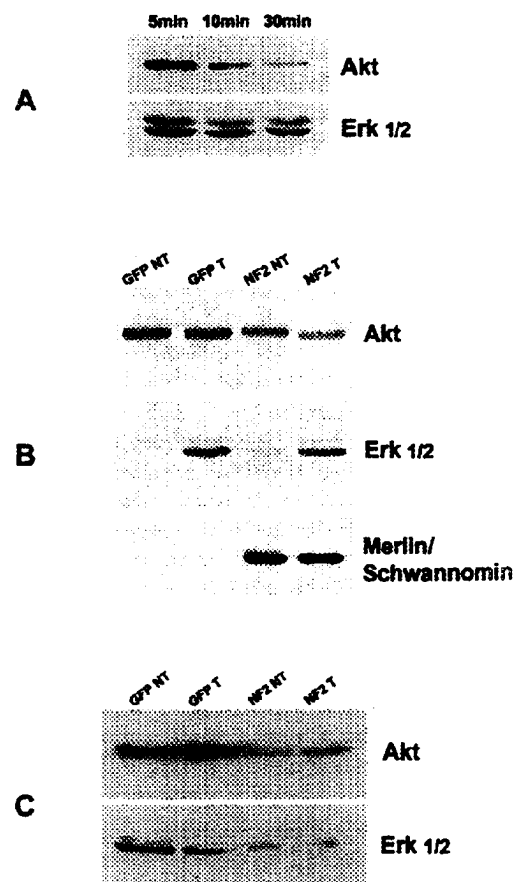

Figure 3. The influence of ligand stimulation on human schwannoma (T, treated; NT, non-treated with the ligand). A, The shortest stimulation with PDGF (5 min) showed the highest activation of Erk 1/2 and Akt. B, In HEI 193 NF2 transduced cells there is a different effect on the activity of Erk 1/2 and Akt to see. In these cells the activity of PDGF-treated ones is drastically reduced compared to GFP-transduced ones. C, The same experiment as in B, with heregulin used as ligand for stimulation of PDGFR internalization.
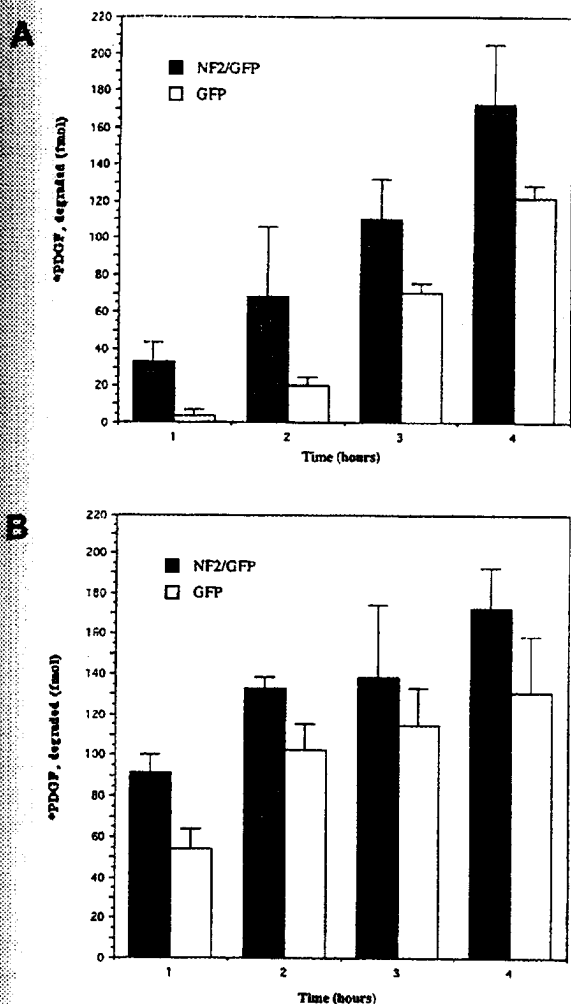
Figure 4. The PDGF-mediated degradation rate of PDGFR in NF2-transduced HEI 193 cells is highly accelerated. Under different conditions (A, plated at 500.000 cells/well and 2 days starvation followed; B, plated at 250,000 cells/ well and 4 days starvation followed) the PDGF stimulation leads to a similar increase of degradation in NF2-overexpressing cells compared to GFP-overexpressing ones.

Acceleration of PDGF-mediated degradation in NF2 over-expressing HEI 193 cells. Since EBP50/NHE-RF is known to be involved in receptor recycling (17), we examined if NF2 overexpression led to significant changes in the internalization of receptor tyrosine kinases (RTK). For these experiments, Merlin/Schwannomin and GFP overexpressing HEI 193 cells were kept under serum-free conditions for up to 4 days. Cells were washed and stimulated with iodinated PDGF for up to 4 h. Fig. 4 illustrates the accelerated degradation of PDGFR in Merlin/Schwannomin overexpressing cells (Fig. 4A, after 2 days starvation, Fig. 4B, after 4 days starvation). A steady increase in degradation occurred, as measured by the amount of degraded 125I*-PDGF in the medium. The GFP-transduced HEI 193 cells showed a slower degradation rate. These results were confirmed using heregulin as ligand for the stimulation of PDGFR internalization (data not shown).

Discussion

Overexpression of PDGFR leads to tumors in many cases (28,29). A PDGF autocrine loop may be causally implicated in tumor development (30). Overexpression of PDGFR may also be involved in tumorigenesis caused by the loss of NF1 gene function in Schwann cell, and meningioma cells in neurofibromatosis 1 (31). The results of this study also indicate that increased expression of PDGFR and EBP50/NHE-RF may play a role in human vestibular nerve schwannomas proliferation (neurofibromatosis 2).

To our knowledge, this is the first demonstration of EBP50/NHE-RF expression in normal nerve Schwann cells and schwannoma tissue. This protein is known to associate with PDGFR (32), and to interact via its first PDZ domain with Merlin/Schwannomin (14). This indicates a role in clustering and location of membrane surface receptors as well as for signaling as for many PDZ-domain-containing proteins was demonstrated (33-37). EBP50 and CFTR are found co-localizing at the apical compartment of epithelial cells (32). Through interaction with the C-terminal motif of cell surface receptors (38), PDZ containing proteins appear to potentiate the signaling pathways of receptors mediated by ligand binding. C-terminal mutants of her-4, PDGFR and serotonin 5-HT2 which have lost the capability to interact with their PDZ-domain protein partners, markedly decrease receptor autophosphorylation (16,39,40). Forming a heteromeric complex between different PDZ proteins has been reported and may explain the similar distribution of different receptors (34). Self-interaction of EBP50 has been reported, although the functional significance of this self-association remains speculative (41). Growing evidence indicates that PDZ-containing proteins participate in trafficking receptors from plasma membrane to endosomal pools, e.g. Cao *et al* (17) found a role for EBP50 in the sorting of β_2-adrenergic receptors. However, since the experiment was conducted in HEK 293 cells which presumably express the NF2 gene, it is not clear whether the EBP50-mediated recycling process is independent of Merlin/Schwannomin in this cell type. Furthermore, it is still obscure whether Merlin/Schwannomin plays any direct role in regulating PDGFR-EBP50 interaction.

The interaction with ERMs and Merlin may be necessary for receptor recycling (14,42). Our study especially investigated the functional aspect of the involvement of the NF2 gene product in RTK-recycling. From results obtained from experiments in *Drosophila* it was proposed, that D-Merlin may mediate proliferation and motility signals in a manner independent of the cytoskeleton, perhaps via an endocytotic process which internalizes growth factor receptors (11).

The role of NF2 in cell proliferation has been investigated extensively (43). Transfection of the NF2 cDNA suppresses a v-Ha-Ras-induced malignant phenotype. Growth suppression of rat schwannoma cells can be achieved by overexpression of isoform I but not isoform II of the NF2 gene (44). Similarly, the phenotype of N-ras transformed cells can be reversed by Merlin expression (43). In this study, we first demonstrated that restoration and overexpression of a functional NF2 gene (isoform II) is able to suppress cell proliferation in a NF2 gene mutated cell line derived from a vestibular nerve
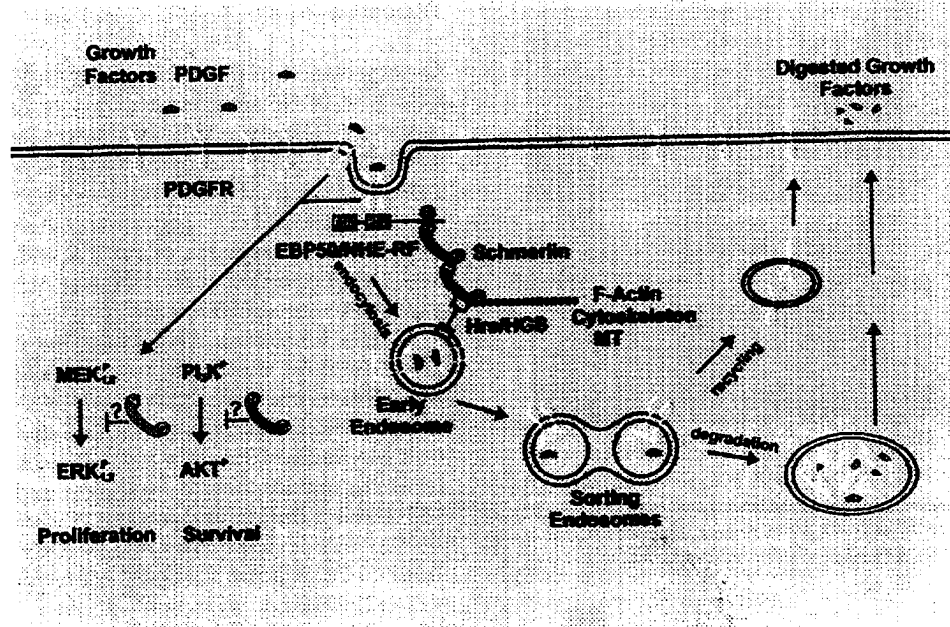
Figure 5. Hypothesis. Our model describes at least two functional effects of NF2 regarding its influence onto receptor internalization (see text).

schwannoma of a NF2 patient. Interestingly, the NF2 growth inhibitory effect could only be observed under serum-free conditions in our *in vitro* system. Shaw *et al* (45) reported that overexpression of Merlin/Schwannomin in cultured mammalian NIH3T3 cells strongly correlated with an increase in cell density and/or serum starvation. Under these conditions, there exists both an unphosphorylated and a serine phosphorylated Merlin/Schwannomin. When serum was re-introduced to the starved cells, the cells rapidly loss the unphosphorylated Merlin/Schwannomin. However, using the same polyclonal antiserum as Shaw used in his study (45), in NF2 transduced HEI 193 cells only a single 66 kDa Merlin/Schwannomin band could be seen regardless of presence or absence of the fetal bovine serum. This suggests that other downstream partner(s) of Merlin/Schwannomin may be phosphorylated by serum, blocking the NF2 mediated growth inhibition in Schwann cells.

The results of this study also suggest Merlin/Schwannomin suppresses the growth of human schwannoma cells, possibly through accelerated degradation of cell surface growth factor receptors. As Cao *et al* demonstrated (17), mutation of the C-terminal with EBP50 interacting portion of β_2-adrenergic receptor, leads to a deficient receptor recycling but not to its degradation. Unfortunately, nothing is reported about the cell proliferation rate for cells containing this mutant. Merlin/Schwannomin also has been found to interact with an early endosome associated protein, hepatocyte growth factor (HGS)-regulated tyrosine kinase (46). HGS/Hrs is a substrate for activated tyrosine kinase receptors, which play a role in endosomal membrane trafficking (47) and is a prerequisite for receptor-mediated activation of Smad2 (48).

We hypothesize a model (Fig. 5) for the functional role of the NF2 gene product. Merlin/Schwannomin may play a bi-functional role in both cell proliferation and growth

inhibition. Through interaction with EBP50/NHE-RF, Merlin/ Schwannomin may accelerate the cell surface receptor recycling. However, Merlin/Schwannomin may also increase the receptor degradation through interacting with HGS/Hrs. In this study, the overexpression of Merlin/Schwannomin in the cells grown in the presence of serum stimulates the cell proliferation, and this suggests that Merlin/Schwannomin may potentiate the cell growth factors recycling through interaction of receptor-EBP50-Merlin.

In summary, our data suggest that: a) PDGFR and EBP50/NHE-RF are overexpressed in human schwannoma tumors and interact *in vivo*; b) schwannoma cells which overexpress NF2 decrease the cell proliferation rate under serum-free condition; c) overexpression of Merlin/ Schwannomin leads to downregulation of PDGF-mediated Erk 1/2 and Akt activation; d) the degradation rate of PDGF is accelerated in NF2 overexpressing HEI 193 cells.

Acknowledgments

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ELSEVIER

Neurofibromatosis 2 Phenotypes and Germ-Line NF2 Mutations Determined by an RNA Mismatch Method and Loss of Heterozygosity Analysis in NF2 Schwannomas

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ABSTRACT: *We used a novel RNase cleavage assay (NIRCA) to screen for neurofibromatosis 2 (NF2) mutations in NF2 schwannomas. Mutations were found in tumors in 16 of 20 patients. Eleven patients (55%) had loss of heterozygosity or loss of one allele, indicating that the mutation was a germ-line mutation. The phenotypes of these patients were consistent with previous NF2 genotype–phenotype correlation studies: patients with nonsense mutations had severe phenotypes, whereas those with splice-site or missense mutations had milder and variable phenotypes. These results confirm the utility of NIRCA as a rapid and convenient method for screening for germ-line NF2 mutations.* © *Elsevier Science Inc., 2000. All rights reserved.*

INTRODUCTION

Mutations in the neurofibromatosis type 2 (NF2) tumor-suppressor gene cause benign nervous system tumors, ocular abnormalities, and skin lesions [1, 2]. Most studies have used polymerase chain reaction–single-stranded conformational polymorphism (PCR-SSCP) analysis [3] or denaturing gradient gel electrophoresis [4] to detect NF2 mutations, but both methods are time consuming and laborious. We used a modified nonisotopic RNase cleavage assay (NIRCA, Ambion, Austin, TX, USA) [5] to rapidly detect NF2 mutations in tumors from NF2 patients. We then examined loss of heterozygosity (LOH) in these tumors and compared phenotypes in patients whose LOH results indicated that the mutations were germ-line mutations.

MATERIALS AND METHODS

Twenty NF2 patients were studied, all of whom met the National Institutes of Health diagnostic criteria [6] and provided informed consent. DNA from schwannomas obtained from normal surgical procedures was analyzed by

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using PCR followed by direct sequencing, with the use of an Applied Biosystems PRISM automated sequencer 310 (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA). Sequencing was used to confirm and characterize NF2 mutations detected at the RNA level by using NIRCA.

The NF2 gene was amplified by reverse transcription PCR (Stratagene, La Jolla, CA, USA) from tumor samples and a normal control. The entire NF2 coding sequence (1785 BP), 88 bp of the 5' UTR, and 54 bp of the 3 ' UTR were amplified from first-strand cDNA by two sets of PCR primers (primer sequences, Genebank accession number Z22664). The NIRCA method modified for NF2 is described elsewhere (paper submitted by R. Faudoa, Z. Xue, F. Lee, M. E. Baser, and G. Hung). Genotyping was performed by using six microsatellite markers (CRYB2, D22S193, NF2CA1, NF2CA3, D22S268, and D22S430).

RESULTS AND DISCUSSION

Substitution, deletion, and insertion mutations were found in tumors from 16 of 20 patients (80%). All mutations were confirmed by direct sequencing, and mutations were not found with direct sequencing in the other four patients. An intronic insertion was found by direct sequencing in patient 97; patient 143's mutation may lie outside the intron–exon boundary regions that were sequenced. Eleven patients with identified tumor mutations had LOH or loss of one allele, indicating that the mutation was the germ-line NF2 mutation (Table 1).

Table 1 Identified germ-line NF2 mutations and phenotypes in NF2 patients

Patient	Altered exon(s)	Sequence change	Codon change	Mutation type	LOH/No. of alleles	Family history	Age at onset	Number of VSs/intracranial meningiomas/spinal tumors
124	2	169 C to T	57 Arg to stop	Nonsense	LOH	S	2	BVS/1/numerous
47	2	185 T to C	62 Phe to Ser	Missense	LOH	F	45	BVS/2/0
92	2	185 T to C	62 Phe to Ser	Missense	LOH	F	14	BVS/1/numerous
143	2–3	NA	114–360 del 246 bp	Splice-site	LOH	S	17	BVS/2/1
13	5	459 C to A	153 Tyr to stop	Nonsense	1	S	5	BVS/0/numerous
133	9	885-5 G to T	811–886 del 75 bp	Splice-site	1	S	18	BVS/0/0
97	10	NA	79 bp ins intron 11	Splice-site	LOH	S	21	BVS/0/several
31	11	1021 C to T	341 Arg to stop	Nonsense	1	S	19	BVS/1/numerous
81	15	1575-1 G to A	1575–1737 del 162 bp	Splice-site	LOH	F	18	BVS/0/0
193	15	1575-1 G to A	1575–1737 del 162 bp	Splice site	1	S	27	BVS/1/3
28	15	1598 A to C	533 Lys to Thr	Missense	1	S	14	BVS/0/3

Abbreviations: LOH, loss of heterozygosity; AS, asymptomatic; BVS, bilateral vestibular schwannomas; S, sporadic; F, familial; SA, splice acceptor site; SD, splice donor site.

Of these 11 patients, those with nonsense mutations had young ages at onset of symptoms (2, 5 and 19 years), and all had numerous spinal tumors. Five patients with splice-site mutations had older ages of onset of symptoms (17–27 years) and fewer spinal tumors (from 0 to several). Three patients with missense mutations had variable ages of onset of symptoms (14 and 45 years), and only one had numerous spinal tumors. The range of number of meningiomas in the 11 patients was relatively narrow (0–2).

Thus, the NIRCA method, when combined with LOH analysis, detected germ-line NF2 mutations in 55% of patients. The phenotypes of these patients were consistent with previous NF2 genotype–phenotype correlation studies [7, 8]. Further applications of the NIRCA assay are likely because the method is well suited to the study of large genes such as NF2, the assay detects pathogenic missense mutations, and assay results are available about 3 hours post-PCR.

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Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2

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Abstract. By using retroviral mediated gene transfer technique, a primary schwannoma culture from a 56-year-old Neurofibromatosis type 2 (NF2) patient was immortalized with HPV E6-E7 genes. This cell line, HEI193, has a unique splice site mutation of the NF2 gene. Both immunocytochemistry and molecular biology techniques were used to demonstrate that this cell line is of Schwann cell origin. Comparison of the primary tumor with HEI193 revealed the same NF2 mutation and an identical pattern of allele loss at multiple loci, indicating that the established cell line had maintained many of the properties of the original tumor. The immortalized cell line was non-tumorigenic in both severe combined immunodeficient (SCID) mice and nude mice, but has altered growth properties such as higher proliferation rate and independence of Schwann cell growth factors. To our knowledge, this is the first attempt to establish permanent cell lines from human NF2 patients. This Schwann tumor-derived cell line may provide a useful model system for the study of familial NF2 tumor pathogenesis, for elucidating NF2 functions and for testing new gene-based therapeutic approaches.

Introduction

Neurofibromatosis 2 (NF2) is a genetic disorder characterized by the formation of multiple benign nervous system tumors, including schwannomas, meningiomas and ependymomas (1-3). These tumors are often benign with slow growth, but their location predominantly within the central nerve system (CNS) may have catastrophic effects on sensitive intracranial and intraspinal structures, thus, causing a high rate of morbidity and mortality. Affected individuals generally develop symptoms of eighth nerve dysfunction, including deafness (often bilateral) and balance disorder (1,4). NF2 occurs with an incidence of approximately one in 40,000 live births where about 50% of cases reveal no family history and are likely due to new mutations (5).

Clinically, there are two subtypes in NF2 patients (5-8). Patients with the severe (Wishart) subtype usually have onset by 20 years of age, develop many CNS tumors in addition to bilateral vestibular schwannomas, and exhibit rapid clinical progression that may lead to death by the third or forth decade (9,10). On the other hand, patients with the mild (Gardner) subtype often do not develop symptoms until the third decade, and have few tumors other than bilateral vestibular schwannomas and have a relatively benign clinical course (5,10).

The NF2 gene is located on chromosome 22q12 (11). The genetic evidence from NF2 patients with schwannomas (12), meningiomas (13), sporadic unilateral vestibular schwannomas (10) and mutated alleles, together with the loss of heterozygosity (LOH) for restriction fragment length polymorphism (RFLPs) on chromosome 22q, has suggested that the NF2 gene is a tumor suppressor gene.

Genotype-phenotype correlation analysis revealed that more severe disease (Wishart) tends to harbor NF2 mutations that result in premature protein termination due to frameshift mutations (deletions or insertions) or the presence of premature termination codon. In the patients with milder clinical disease (Gardner), the mutations are either missense mutations or due to not being found by ordinary mutation detection methods. It has been predicted that nonsense NF2 mutations could result in unstable truncated proteins whereas missense mutations might generate full-length proteins incapable of growth suppression (14). In a recent report, differing growth rate were noted in culture when different sporadic vestibular schwannoma tumor tissues were cultured (15). Unfortunately, no NF2 gene mutation information was provided.

Currently, several biochemical experiments have also indicated that the NF2 protein directly interacts with plasma membrane molecules such as CD44, EBP50 or hNHE-RF/

EBP50 and cytoskeleton molecules such as ß II-spectrin (16). These findings suggest that the NF2 gene product may, like its homologous proteins ezrin, radixin and moesin (ERM), work as a linker between cell membrane and cytoskeleton. This linkage may block cytoplasmic growth signaling from the cell surface. However, a detailed pathway of how NF2 protein mediates the tumor suppressor function is still unclear.

The establishment of an NF2-*in vitro* model is essential to elucidate the NF2 gene tumor suppressor function in Schwann cells and as the first step to test new therapeutic approaches. To date, no single cell line has been developed from NF2 tumor cells, and most studies were conducted either in yeast, mouse schwannoma cells or other non-Schwann human cells. There are three main reasons for this limited progress. First, human Schwann cells are difficult to obtain. Second, because of the lack of knowledge of Schwann cell growth factors, once the Schwann cells are obtained, they do not proliferate in culture. Third, there is the contamination of human fibroblast. Recently, we have developed a method for establishing short-term primary schwannoma cells in culture. These primary cultures can be enriched to greater than 99% pure and could be very useful for the study of genetic alterations and NF2 gene functions at the cellular and molecular level (17). In this report, based on the primary cultures, we took additional steps for the first time to establish and characterize a stable long-term schwannoma cell line. The establishment of such a cell line provides another method to study tumor growth regulation conferred by NF2 and could also serve as a useful tool to test the gene-based therapeutic approaches in a disease model.

Materials and methods

Patient material. The patient was a 56-year-old male with an NF2 tumor of the ears. Neither his parents nor siblings have any features suggestive of NF2. His only 27-year-old daughter does not have any certain features of NF2. He had bilateral vestibular schwannomas and right optic nerve tumor acoustic at the age of 39. When he was 40, he underwent posterior fossa craniectomy for removal of the right vestibular schwannoma, which left him deaf on that side. Subsequently, he underwent the surgical removal of a meningioma which included exenteration of the globe. Two years ago, the patient underwent surgical removal of the left vestibular schwannoma. After obtaining informed consent for genetic studies, blood and tumor tissue were obtained from the patient. No adjuvant chemotherapy or radiation had been given prior to collection of tumor material.

Tumor cell culture establishment
Primary culture. The detailed procedure for establishing primary vestibular schwannoma culture was described previously (18). In brief, vestibular schwannoma tumor tissue from the patient was transferred to the laboratory and cut to 2 mm^3 in size and digested with 10 mg/ml collagenase/dispase (Boehringer Mannheim) at 37°C with 5% CO_2 for one hour. The digested tumor cells were washed and transferred twice during the first 48 h, and then plated in 100 mm laminin-coated dishes with modified D-MEM/F12 medium (Gibco/BRL)

supplemented with insulin 10 µg/ml (Sigma), progesterone 3×10^{-8} M (Sigma), heregulin 10 nM (Genentech), bovine pituitary extract 3 µg/ml (Gibco/BRL), transferrin 10 µg/ml (Sigma), α-tocopherol 5 µg/ml (Sigma) and forskolin 5 µM (Sigma).

Immortalization of primary culture. At the first passage, 5×10^5 cells were plated in 60 mm laminin-coated plate, and fetal bovine serum (FBS) (HyClone, Utah) was added to 10%. Twenty-four hours after the serum was added, 1×10^7 of retrovirus construct pLXSN containing human papilloma virus (HPV) E6-E7 genes and Neo^r gene were added to the culture medium (19-21). The culture cells were then maintained at 37°C with 5% CO_2. Once colonies were formed, G418 (Gibco/BRL) was added to the final concentration of 0.4 µg/ml for neomycin resistance selection. After 8-10 days and several changes of the culture medium, the drug resistant cells were recorded as passage 0. The cell line was designated HEI193 (for House Ear Institute).

Proliferation assays. Proliferation of the cells was monitored by visualizing 5-bromo-2'deoxyuridine (BrdU) incorporation (22) using colorimetric immunoassay kit (Boehringer Mannheim), according to the manufacturer's recommendation.

Immunohistochemical characterization. The cultured tumor cells were plated in laminin-coated chamber slides. Forty-eight hours later, plated cells were fixed in methanol/ethanol (1:1) at -20°C for 15 min. The slides were then washed three times with phosphate-buffered saline (PBS) and blocked with 10% normal goat serum (NGS) in PBS for 20 min at room temperature. Cells were incubated for one hour with primary antibody - S100 (1:500, Dako Corp., Carpenteria, CA) which is a marker specific for Schwann cells among peripheral nerve cell types. After washing three times with PBS, secondary fluorescein-isothiocyanate (FITC) conjugated goat anti-rabbit antibody (1:15, Dako Corp.) was added and incubated for 30 min at room temperature. After three washes with PBS, the slides were covered with antifade/pipidien iodine or regular permount and viewed under a Zeiss fluorescent microscope (Zeiss, Germany).

RT-PCR and DNA sequencing. To detect and compare NF2 gene messages from HEI193 cells, patient tumor tissue and patient blood, RNA was prepared by using TRIzol solution (Gibco/BRL) according to the manufacturer's protocols. Reverse-transcription polymerase chain reactions (RT-PCR) were carried out using a RT-PCR kit (Stratagene), according to the manufacturer's procedure. The primers used to amplify the entire NF2 coding sequence from the RNA-derived cDNAs were: sense primer: 5'-ATGGCCGGGGCCATCGC TTC-3', antisense primer: 5'-GAGCTCTTCAAAGAAGGCC ACTC-3'. The PCR profile was as follows: 94°C for 2 min, 68°C for 3 min for 30 cycles. The final extension was added at 68°C for 10 min before storage at 4°C. The PCR products from tissue, blood, and HEI193 cells were loaded onto a 1% of agarose gel for electrophoresis. For direct DNA sequencing of the PCR products, seven different primers were synthesized for sequencing the entire coding region (17). The sequencing reaction procedure was according to
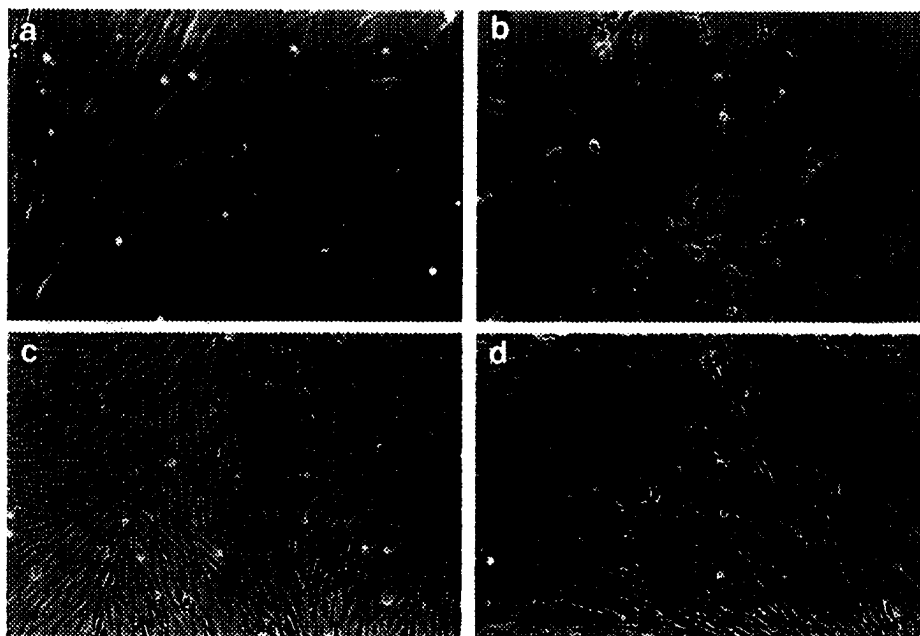
Figure 1. Morphology of primary and immortalized schwannoma culture cells: (a), Primary schwannoma cells at passage 1 from which HEI193 cell line was derived. (b), A colony has formed 4 weeks after the viral transduction. The immortalized schwannoma cells are arranged in a pattern of whorls, strands and sheaths in confluent condition [(c), 90%; and (d), 100%]. (x100).

the manufacturer's instructions (ABI Big-Dye Terminator Perkin-Elmer, CA), and the reactions were run and analyzed by an automated Genetic Analyzer ABI 310 system.

Karyotyping analysis. Karyotyping was performed by Dr B. Hukku at Children's hospital of Michigan at Detroit, when HEI193 cell line at passage 17. In brief, the logarithmic phase of cell growth were arrested with 0.06 µg/ml of colcemid for 1 h, and then trypsinized and treated with 0.0375 M of KCl for 9 min. The cells were fixed with a mixture of methanol/acetic acid at a 3:1 ratio (23). Cell suspensions in the fixatives were dropped onto cold wet glass slides. The slides were air dried and stained with 4% Giemsa solution. Chromosomes were examined and counted to establish the ploidy distribution and constitutional aberrations. For trypsin Giemsa banding of chromosomes, the slides were aged at 60°C on a slide warmer for 18 h, trypsinized for 3 sec, stained with Giemsa stain for 11 min (24), washed, dried and then mounted in permount. The well-banded metaphases were karyotyped using AKSII image analysis system.

Loss of heterozygosity (LOH) analysis. For loss of hetero-zygosity analysis, genomic DNAs were extracted from the corresponding tumor tissue, blood and cultured HEI193 cells by using TRIzol solution (Gibco/BRL). LOH analysis was performed using five selected microsatellite markers either flanking or within the NF2 gene: CRYB2 (centromeric) (25), D22S275 (centromeric) (MacCollin, unpublished data), NF2CA3 (intragenic) (26), D22S268 (telomeric) (27) and D22S430 (telomeric) (28,29). These five markers were amplified from tumor tissue, cultured cells or blood DNAs and analysed for their heterozygosities. One of each primer

pair was fluorescently labeled with either one of three dye phosphoramidites - FAM, TET or HEX (ABI, Foster City). PCR amplifications and electrophoresis were performed as described previously (30). The amplified markers were separated on an automated Genetic Analyzer ABI310.

Inoculation of schwannoma cells in mice. Three of each SCID (severe combined immunodeficient) mice and nude mice were used in this study. 6x10⁶ of HEI193 cells were subcutanously injected into right flank area of the mice. The mice were followed up to 8 weeks for signs of tumor growth.

Results

Cell line establishment. Vestibular schwannoma tissue, which has been characterized containing a unique NF2 mutation (1575-1 G to A) at exon 15 splicing site from a 56-year-old NF2 patient (30), was used for isolating the schwannoma cells. At the first passage, the cells were immortalized with a retrovirus construct pLXSN encoding HPV E6-E7 and Neo^r. After 8-10 days of G418 selection, characterization of the cell line was performed. The cell line was designated HEI193 for House Ear Institute and has been propagated in culture for over 40 passages.

Morphology changes. Four to six weeks post retroviral transduction of the primary culture, a small population of short bipolar, spindle shape cells have formed colonies mixed in the untransduced schwannoma cells with long process shape (Fig. 1a and b). Interestingly, only during the first 7 passages, when in confluence condition the cell were growing arranged like *in vivo* which formed a pattern of
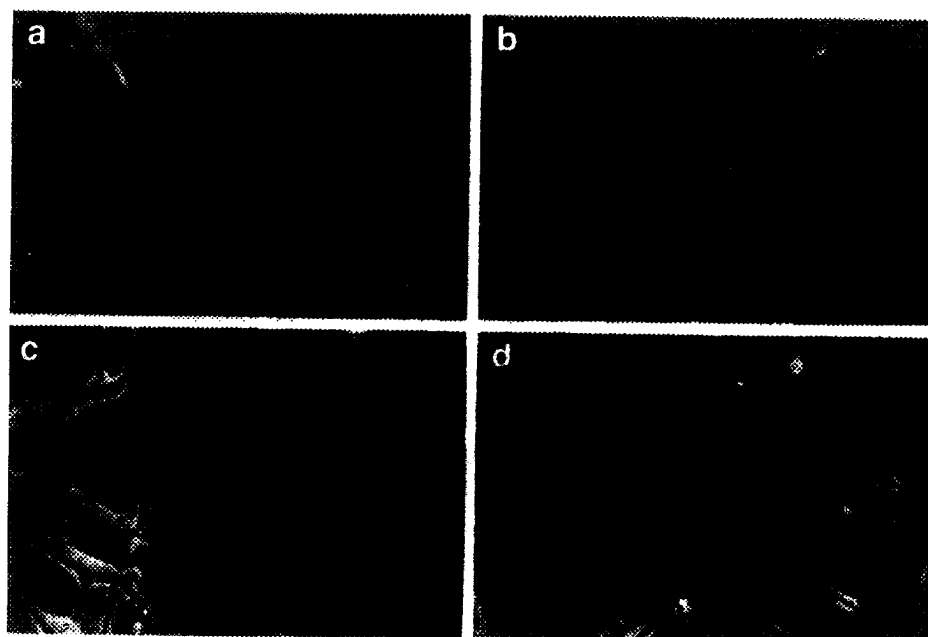
Figure 2. Morphology change of HEI193 cell line during the immortalization process. (a), Shows more and more multinuclear syncytium cells could be seen after Passage 7. (b), The multinuclei as well as cytoskeleton elements were clearly displayed after Passage 14. (x200). (c), The cells displayed the heterogeneity with different shapes at Passage 9. (x100). (d), Shows that the cells at Passage 15 displayed more homogenous shape. (x100).
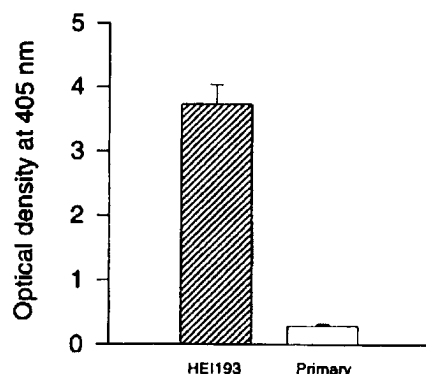

Figure 3. A comparison of proliferation rates of HEI193 cells and its parental primary culture cells.
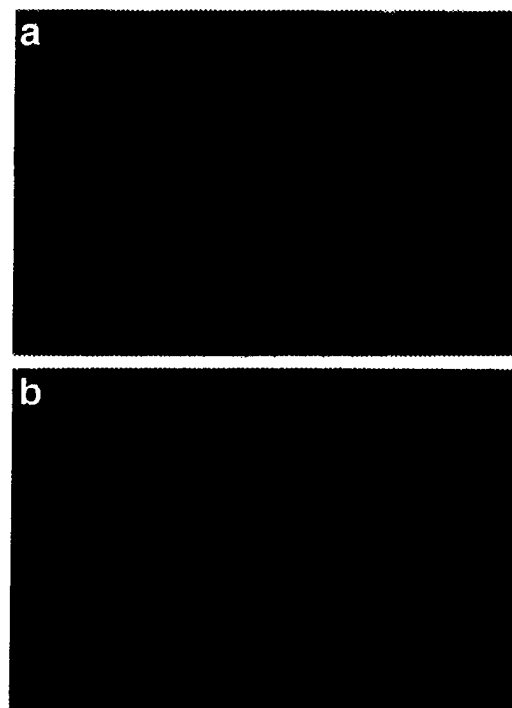

Figure 4. Immunohistochemical staining of the culture for Schwann cell specific antibody S100. Different S100 expressions were observed of the culture cells at passage 7 (a) and passage 17 (b) (x100).

whorls, strands and sheaths (Fig. 1c and d). After passage 7, the cultured cells started to show increasing heterogeneity in morphology and decreased proliferation rate. More and more multinuclear syncytium cells could be seen in the culture (Fig. 2a and b). It is interesting to note that in some of the syncytia the cytoskeleton elements were distinctly displayed (Fig. 2a and b). At passage 14 the culture reached its peak of crisis during which approximately half of cells began to die. This phenomena lasted about two weeks at which time a distinguishable small population of the culture cells with even shorter spindle shape survived and grew well (Fig. 2c and d).

Growth rate. The transduced cells have a dramatic increase in growth rate. Proliferation rate of HEI193 cells was measured by using colorimetric immunoassay (22). The BrdU incorporation in HEI193 cells at passage 17 was 13 times (3.730/0.29 of OD405) more than that in primary cells (Fig. 3).
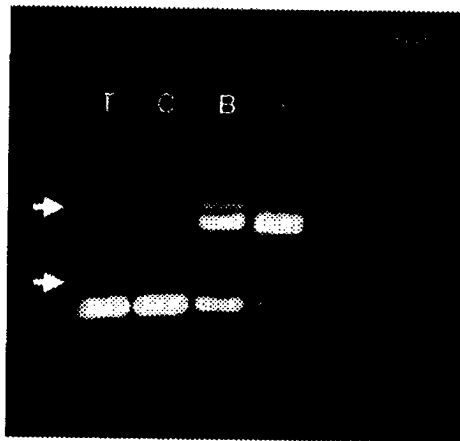
Figure 5. NF2 gene analysis of HEI193 cells. RT-PCR products of NF2 gene from tumor tissue (T), HEI193 cultured cells (C), the patient's blood (B) and (*), the positive control NF2 cDNA were loaded onto a 1% agarose gel for electrophoresis. The far right lane and the number column is molecular size marker. It is noted that except the truncated NF2 transcripts were detected (lower bands), no wild-type NF2 message can be detected from the cultured cells. Both wild-type and mutated transcripts were observed in RNA extracted from tumor tissue and blood samples. The big arrowheads show PCR products containing alternative splicing with exon 16. The small arrowheads point out the PCR products without exon 16.

Immunohistochemical staining. To assure that the cells purified and expanded from schwannomas were Schwann cells, the characteristics of the primary culture cells were stained with Schwann cell marker S100 (17). Greater than 99% of schwannoma cells were used for immortalization. The culture cells at every other passage were stained for Schwann cell

specific antibody S100 which shown increasing heterogeneous expression during immortalization (Fig. 4).

NF2 gene analysis. To detect and compare NF2 messages from the cultured cells, patient tumor tissue and patient blood, RT-PCR products of 3' end of NF2 gene from the same patient were electrophoresed on an agarose gel (Fig. 5). Both PCR products from tissue and blood showed the wild-type and mutated NF2 gene messages. PCR products from tumor tissue showed more than 90% of the products were the truncated form and less than 10% of WT NF2 transcript from normal stroma tissues. In blood, the wild-type/mutant-PCR products ratio is about 50:50. In contrast, the PCR product from the cultured cells contained only the mutated NF2 messages. This further confirms that HEI193 cell line has no contamination of other non-tumor cells. By direct DNA sequencing, the PCR products from both tumor tissue and cells of HEI193 cell line showed the same splice site at the acceptor site of exon 15, suggesting HEI193 retained the patient schwannoma origin.

Chromosomal analysis. According to chromosome count, the HEI193 cell line is hypodiploid human male (XO), with most chromosome counts in the 35-41 range. The model number was 37. There were about 75% hypodiploid and 25% hypo-tetraploid metaphases observed. The composite karyotype was: 35-41, XO -(8, 9, 9, 13, 13, 14, 15, 17, 18, 19, 20 and 22) + [t (9, 13)(q10, q10) = M1, add (9)(q33) = M2, add (14)(q10) t(14;20)(q10,p10)=M3, t(15;?)(q10;?) = M4, der (19) t(13,19)(q12,q13) = M5] -(6, 10, 15, 17, 21) X15. There was loss of one complete copy of chromosomes 8, 17, 18 and 22 in the majority of metaphases (Fig. 6).
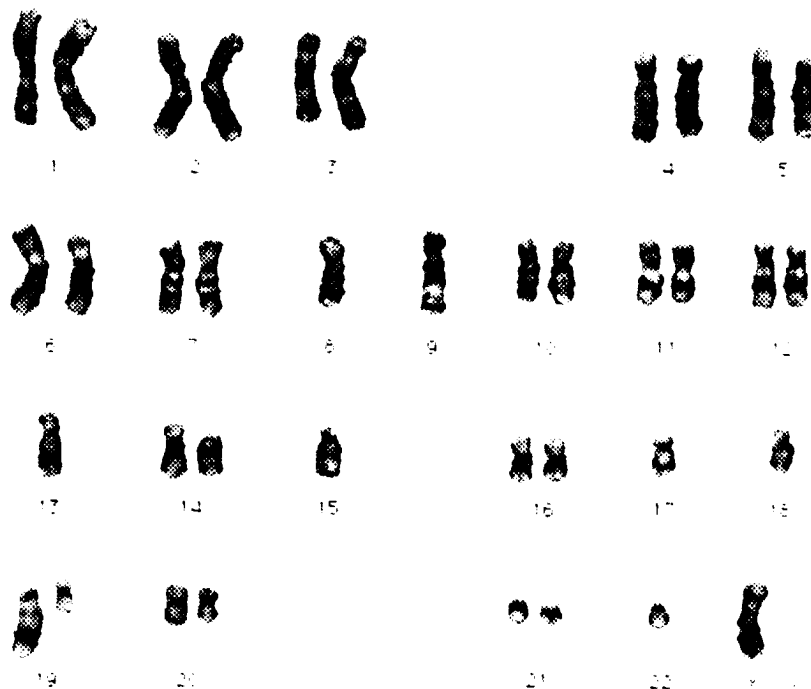

Figure 6. Karyotype of metaphase cell from HEI193 cell line. One complete copy of chromosomes 8, 13, 15, 17, 18, 22 and Y has been lost in majority of metaphases.
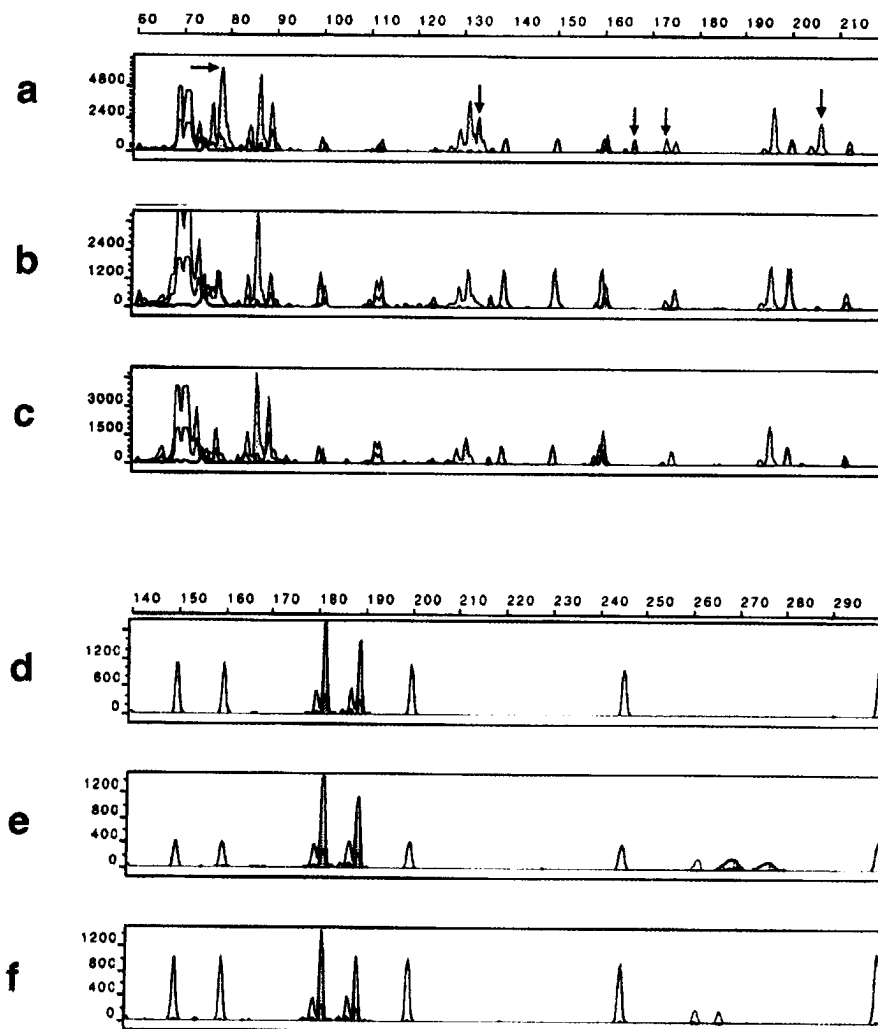
Figure 7. LOH analysis of blood, tumor tissue and HEI193 cell line. The electrophoregrams show 5 microsatellite markers [D22S430 (Peak 79, 87), NF2CA3 (Peak 131, 133), D22S275 (Peak 161, 167), CRYB (Peak 173, 175) and D22S268 (Peak 196, 206)]. Comparing with the electrophoregram display from blood DNA (a), LOH were observed in DNAs from both (b), tumor tissue and (c), HEI193 cells. The arrow bars point to the peaks present in the blood DNA, but missing in the tumor tissue and culture cells. To examine any large fragment of intra-chromosomal deletion, the same kind of LOH analysis for chromosome 17 was also performed with the DNA from (d), blood; (e), tumor tissue; and (f), HEI193 cell line. No significant loss of heterozygosity was observed on chromosome 17 except total loss of second chromosome 17 was demonstrated in Fig. 6.

Loss of heterozygosity analysis. Genomic DNAs were extracted from primary culture cells and cell line HEI193 at passages 6 and 9 and compared with DNA from the peripheral blood cells as well as tumor tissue. LOH analysis using microsatellite markers flanking or within the NF2 gene revealed loss of some parts of NF2 allele in tumor tissue and cultured cells, but not in blood cells. In the blood sample, the heterozygosity showed two peaks for each of the markers: D22S430 (peak 79, 87), NF2CA3 (peak 131,133), D22S275 (peak 161, 167), CRYB (peak 173, 175) and D22S268 (peak 196, 206). In contrast, the DNAs from either tumor tissue or the HEI193 cell line showed only one heterozygosity peak out of two in each marker (Fig. 7a, b and c). This suggested that the second somatic mutation in tumor cells is either a large deletion on the trans chromosome or loss of the entire copy of chromosome 22.

Tumorigenicity in mouse. No sign of tumor growth was observed in either SCID (n=2) or nude mice (n=2) during the 8 weeks post-innoculation of schwannoma cell line.

Discussion

By using retroviral mediated gene transfer technique, we have described the establishment of vestibular schwannoma from neurofibroma type two (NF2) patient with a unique NF2 gene splice site mutation. The clinical manifestations of this patient were typically a mild phenotype. According to our knowledge, there is no report on the successful establishment of a human schwannoma cell line. Until recently, because of the availability of a Schwann cell growth factor, heregulin, our laboratory and others (15,17,30-32) have used a similar method for establishing short-term primary schwannoma

cells in culture. The ideal *in vitro* system to study NF2 at the cellular and molecular level and to test therapeutic approaches is primary cell culture because it best represents *in vivo* characteristics. However, most of the surgical specimens are too small for sufficient cell numbers, and once the tissue cells grow in culture they usually stop proliferation in early passages, even cultured in the chemical defined medium which subsidizes several Schwann cell growth factors and mitogens such as insulin, heregulin and forskolin. There may exist some key factors which could keep the culture cells proliferating and which have not yet been identified. In addition, primary culture cells are polyclonal and more or less contaminated with other stroma cells. All of these factors may affect both the quality and reproducibility of research. Establishing stable immortalized Schwann and schwannoma cell lines is essential.

In this study, we have attempted to immortalize vestibular schwannoma cells from a primary Schwann cell culture. We have chosen a retroviral mediated gene transfer technique to deliver the immortalization agent. Retrovirus has relatively higher transduction efficiency than non-viral carriers, and most importantly the retrovirus can stably integrate into the target cell genome. Human papilloma virus type 16 E6 and E7 genes are considered as oncogenes which have been found associated directly with cervical cancer in women (33). It is found that at molecular level the E6/E7 gene products interfere with the function of tumor-suppressor protein p53 and Rb (retinoblastoma), respectively, thereby preventing cell cycle arrest without causing significant transformation (34,35). Transduction of either E6 or E7 gene alone can prolong the primary culture life by inducing hyperproliferation, but has very low frequency of transduction in primary cells. However, by co-expression of E6 and E7 genes, we can substantially increase the immortalization events (36,37).

In general, a two-stage model of mortality stage 1 (M1) and mortality stage 2 (M2) is involved during immortalization process (37). The M1 leads to cellular senescence, when normal cells have reached the end of their proliferation capacity. Expression of HPV E6/E7 proteins can help cells overcome M1. Escape from M1 results in an expanded *in vitro* life span during which the cells continue to proliferate until M2. At this point most cells enter crisis. Eventually, some cells gain additional genetic alteration that allows them to survive at M2 and have ability to proliferate indefinitely (e.g., immortalization). The same phenomena have been recently reported when using HPV E6/E7 to immortalize human bronchial epithelial cells (37). We have observed two major phenotypic changes during the immortalization process. The first phase is between passages 1 and 14. Between passages 1 and 7, the increasing proliferation rate and life span were observed in culture. M1 began at passage 7 and reached its peak at passage 14, at which time cells exhibited increasing heterogeneity in shape and cell death. A population of cells formed syncytia, which is believed to be the signal of cell death (17). By the second phase, at passage 15, a population of cells had gone through the crisis and survived to become immortalized. By comparing the genotyping data from primary culture cells at passages 6 (data not shown) and 9 with the blood and the tumor tissue, no sign of LOH in chromosome 17 was observed (Fig. 7d, e and f). But when

the cells were karyotyped at passage 17, loss of one copy of chromosome 17 in addition to other monosomies, chromatid gaps and breaks and aberrant chromosome has been observed (Fig. 6). Similar high genomic instability and telomerase activity have also been seen in human bronchial epithelial cell immortalization by E6 and E7 genes (37).

The Schwann cell origin of the immortalized cells was confirmed by the presence of Schwann cell marker S100 (Fig. 4). Although the immortalized culture is still a pool of transformed cells, RT-PCR has confirmed no wild-type NF2 gene transcripts were detected (Fig. 4) from the cells. Furthermore, loss of polymorphic marker or of whole copy of chromosome 22 was observed in cytogenetic data, suggesting that this tumor has a splice site point germline mutation. The immortalized schwannoma cells have increased population doubling rate, high saturation density and altered expression of S100. However, the genetic situation at the current passage still does not complete the requirement of tumorigenicity in animal. Continuous proliferation of immortalized cells may accumulate further genetic alterations, which may eventually transform the cells to be completely tumorigenic.

In conclusion, our results demonstrate for the first time that it is possible to establish a human schwannoma cell line by immortalization with a retrovirus construct containing human papilloma virus E6/E7 genes. This immortalized schwannoma cell line, HEI193, is stable and non-malignant with Schwann cell origin and NF2 gene mutation. This cell line will be very useful for studying NF2 gene function, genotype phenotype correlation of NF2, mutagenesis and tumorigenicity of schwannoma cells. In addition, it is potentially an *in vitro* model for testing therapeutic approaches.

Acknowledgments

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Immunohistochemistry Study of Human Vestibular Nerve Schwannoma Differentiation

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ABSTRACT   Differentiation of primary human vestibular nerve schwannomas (VS) caused by mutations of the NF2 gene was evaluated by examining the expression patterns of genes that are specifically expressed in different stages of Schwann cell lineage. In schwannoma cells that are not in contact with an axon, the expression levels of the major myelin sheath proteins, such as protein zero glycoprotein (P0), myelin basic protein (MBP), and peripheral myelin protein 22 (PMP22), were greatly reduced. However, high expression levels of nerve growth factor receptor (NGFR), neural cell adhesion molecule (N-CAM), and cell adhesion molecule L1 (L1) were observed. In addition, expression of transcription factors Krox20, Krox24, and SCIP/Oct6 was also detected in the tumor cells. These results suggest that loss of the NF2 gene was responsible for the transformation of the Schwann cells into a neoplastic stage that has a similar genetic profile to the pro-myelinating stage. Finally, the primary human vestibular schwannoma cells failed to be regulated and redifferentiated by a regenerating axon, when the human tumors were transplanted into sciatic nerve of nude rat. These results suggest that the NF2 gene might be involved in the differentiation of Schwann cells. *GLIA 38: 363–370, 2002.* © 2002 Wiley-Liss, Inc.

INTRODUCTION

Schwann cell proliferation and differentiation are highly regulated processes that occur only during development and nerve regeneration. These processes are thought to be mediated through the interactions of axon and Schwann cells (Scherer, 1997). The interaction between the axon and the Schwann cell is also essential for determining whether a Schwann cell will differentiate into a myelinating or nonmyelinating type. Several transcription factors, such as SCIP/tst-1/Oct-6, Krox20, and Krox24, which are modulated by the axon, play roles in the differentiation and myelination of Schwann cells (Scherer, 1997; Topilko et al., 1997; Arroyo et al., 1998; Warner et al., 1998, Zorick et al., 1999). Studies in mice have demonstrated that high levels of SCIP, Krox20, myelin glycoprotein zero (P0), myelin basic protein (MBP), galactocerebroside (GalC),

and peripheral myelin protein 22 (PMP22) are expressed in the late pro-myelinating cells, as well as in myelinating Schwann cells (Blanchard, 1996). In contrast, another set of transcription factors, Pax-3, c-jun, and Krox 24, as well as other genes, such as nerve growth factor receptor (NGFR), growth-associated protein-43 (GAP-43), and neural cell adhesion molecules N-CAM and L1, are turned on in immature, pre-myelinating, and denervated Schwann cells (Kioussi et al.,

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1995; Nikam et al., 1995; Stewart 1995; Shy et al., 1996; Vaudano et al., 1996).

The proliferation of Schwann cells ceases once the appropriate number of cells has been generated to encase the entire length of the peripheral nerve. Schwann cells can, however, reenter the proliferative cycle as a result of trauma or tumor formation. Two types of Schwann cell-derived tumors arise when growth control is lost in peripheral nerves in humans: neurofibromas and schwannomas. Neurofibromatosis type 2 (NF2) is an inherited genetic disorder of the nervous system that affects about 1 in 40,000 persons. NF2 is characterized by the presence of bilateral vestibular schwannoma (VS), a benign tumor of the vestibular portion of the eighth cranial nerve. NF2 patients may also suffer from meningiomas, other CNS tumors, including spinal cord tumors, and cataracts. Although bilateral VS occurs only in NF2, unilateral VS occurs sporadically and comprises approximately 90% of all VS cases. It is estimated that VS accounts for approximately 8–10% of all intracranial tumors and nearly 80% of all cerebellopontine angle tumors (see Nager, 1985, for review).

Mutations in the NF2 gene have been detected in approximately 70% of VS. The gene product of the NF2 gene, Merlin/Schwannomin, is thought to act as a tumor suppressor and is believed to be the primary cause of NF2 (Rouleau et al., 1993; Trofatter et al., 1993; Jacoby et al., 1994; Gutmann et al., 1997; Stemmer-Rachamimov, 1997). In vitro studies have suggested that Merlin/Schwannomin interacts directly with plasma membrane-related molecules such as CD44 (Sainio, 1997; Sherman, 1997), Na^+-H^+ exchange regulatory cofactor, hNHE-RF/EBP50 (Murthy et al., 1998; Reczek and Bretscher 1998), as well as with the cytoskeletal protein Fodrin/β II-spectrin (Scoles et al., 1998). There is evidence of the tumor suppressor function of Merlin/Schwannomin through interaction of cytoskeleton elements. It has been suggested that actin plays an important role in the myelination process (Trapp et al., 1989; Kelly et al., 1992; Trapp et al., 1995; Fernandez-Valle et al., 1997). Disorganized cytoskeleton was observed in NF2 mutated schwannoma cells, but not in normal Schwann cells (Pelton et al., 1998). Treating the cultured Schwann cells with cytochalasin D inhibits the expression of myelin-associated glycoprotein (MAG) and protein zero (P0) (Fernandez-Valle et al., 1997). Moreover, mutation of a mouse cytoskeletal crosslinker protein gene, dystonin, led to downregulation of both P0 and PMP22, resulting in a demyelinating neuropathy in mice (Bernier et al., 1998). Furthermore, another Schwann cell tumor suppressor gene, NF1, which acts as GTPase activating protein, was shown to be required for the myelination process (Rosenbaum et al., 1999).

No study has described the cellular phenotypes of human vestibular schwannomas. The NF2 gene is expressed ubiquitously in most cell types throughout the body (Trofatter et al., 1993). It remains unknown whether (1) Merlin/Schwannomin expression is regulated through the interaction of Schwann cells and axons, or (2) Merlin/Schwannomin is required during the transition from the proliferative pro-myelination stage to myelination stage.

In the present study, we hypothesize that the loss of NF2 gene function led to transformation and dedifferentiation of Schwann cells from a myelinating stage to a nonmyelinating stage. To test this hypothesis, we attempted to define the molecular lineage of human VS cells through analysis of the expression of myelin sheath proteins (P0, MBP, PMP22, MAG), transcription factors (SCIP/tst-1/Oct-6, Krox20, Krox24), and neural cell adhesion molecules (N-CAM, L1). Our results demonstrate that transformed VS cells express a similar set of proteins as pre-myelinating and pro-myelinating denervated Schwann cells. After transplantation of human vestibular schwannoma cells into immunodeficient nude rats, we also found that NF2 mutated schwannoma cells fail to be regulated by the regenerating nerve. These results suggest that the NF2 gene may play a role in Schwann cell differentiation from pro-myelinating to myelinating cells.

MATERIALS AND METHODS
Human Vestibular Schwannoma Tumors

Fresh human vestibular tumor tissues removed from the patient were placed immediately in liquid nitrogen, transported to the laboratory, and kept in a −80°C freezer.

Immunohistochemistry

Frozen sections (5 μm) were obtained using a cryostat at −24°C. The sections were fixed for 15 min immediately after cutting; 10% neutralized formalin was used for all sections to be stained for nuclear proteins, and 100% acetone was used for sections to be stained for cytoplasmic and membrane proteins. The slides were washed three times in phosphate-buffered saline (PBS) and were blocked with 0.3% H_2O_2 for 15 min at room temperature (RT). They were then washed twice with PBS for 5 min each and blocked with 10% normal goat serum (NGS) at RT for 20 min. The primary antibody was then added at the appropriate dilution in NGS (Table 1) and incubated for 1 h at RT. The slides were washed three times with PBS, followed by 30-min incubation of horseradish peroxidase (HRP)-labeled goat anti-mouse or anti-rabbit antibody (Dako). The slides were washed again three times with PBS and covered with DAB for 2 min. Finally, the slides were counterstained with 1% methyl green, dehydrated with butanol and xylene, and visualized.

Western Blotting

The concentration of protein extracts dissolved in 1% sodium dodecyl sulfate (SDS) was measured with the

TABLE 1. Antibodies Used

Antibody	Dilution	Vendor	Source	Fixation
P0 (human specific)	1:200	Gift from Dr. Schachner, Germany	Mouse	95% ethanol
P0 (mouse and human)	1:1,000	Gift from Dr. Archelos, Austria	Mouse	95% ethanol
L1	1:40	Transduction Laboratories, KY	Rabbit	95% ethanol
SCIP	1:300	Gift from Dr. Wegner, Germany	Rabbit	Formalin
Krox20	1:50	Berkeley Antibody Company	Rabbit	Formalin
Krox24	1:50	Santa Cruz Bio, CA	Rabbit	Formalin
MAG	1:100	Gift from Dr. Quarles, National Institutes of Health, Bethesda, MD	Mouse	95% ethanol
N-CAM	1:50	NeoMarkers, Union City, CA	Mouse	95% ethanol
GalC	1:20	Chemicon International	Mouse	95% ethanol
GAP-43	1:35	Novocastra Laboratories	Mouse	95% ethanol

P0, protein zero; MAG, myelin-associated glycoprotein; N-CAM, neural cell adhesion molecule; GalC, galactocerebroside; GAP-43, growth-associated protein-43.

BCA assay kit (Pierce, IL). In this study, 25 μg of total protein was separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, IL) in Towbin buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). The membrane was blocked with 7% nonfat dry milk in 0.1% Tween-20 in PBS (PBS-T) and washed with PBS-T. Primary antibodies were incubated with the membrane for 1 h and then washed with PBS-T. The membrane was then incubated with goat anti-rabbit antibody coupled with HRP for 45 min and subsequently washed by PBS-T. The presence of secondary antibody bound to the membrane was detected by ECL (Amersham).

Human Wallerian Degeneration Model and Primary Human Vestibular Nerve Schwannoma Implantation

Establishment of human wallerian degeneration model

In an effort to compare the gene expression profile differences between schwannoma cells and demyelinating Schwann cells during injury, we developed an animal Wallerian degeneration model by transplanting fresh human sciatic nerve tissues into nude rats. Fresh collated human sciatic nerve tissues were dissected to 4×20 mm³ size. Five-week-old female nude rats were anesthetized with ketamine (60 mg/kg) combined with xylazine hydrochloride (10 mg/kg) by intraperitoneal injection. A 2.5-cm incision was made at both sides of the flank area, and the nerve tissues were placed subcutaneously. The wound was closed with wound clips. The animals were sacrificed at 6 weeks post-implantation. The implanted nerve tissues were removed, fixed, sectioned, and immunostained.

Primary human vestibular nerve schwannoma implantation

This experiment examined whether the NF2 mutated schwannoma cells were able to redifferentiate and myelinate the axon. Immunodeficient nude rats were used to study interactions of vestibular nerve schwannoma and growing axons. Primary human vestibular nerve schwannomas freshly removed from NF2 patients and normal human sciatic nerve were collected and expanded in different cultures (Hung et al., 1999). Two weeks after expansion in culture, the purity of the cells were immunostained by S-100, a marker for both normal Schwann and schwannoma cells. More than 95% Schwann cell cultures were dissociated by trypsin and mixed with Matrigel (Collaborative Research, Bedford, MA), in a ratio of 30:70 (v/v) with DMEM, so that the final cell concentration equaled 120×10^6/ml. The cells were then injected into a 0.7-mm internal diameter PAN/PVC tube (gift from Dr. Aebischer). A 6-mm segment of the filled tube was cut and capped with superglue and kept overnight in DMEM at 37°C. The 5-week-old female nude rats were anesthetized with ketamine (60 mg/kg) combined with xylazine hydrochloride (10 mg/kg) by intraperitoneal injection. The right sciatic nerve was exposed using a muscle-splitting incision of the posterolateral thigh musculature. A 5-mm segment of the sciatic nerve was removed at the middle thigh and sutured with the cell-filled tube. The wound was then closed in layers. The animals were sacrificed at 4 weeks post-implantation. The implanted tube plus 3 mm of sciatic nerve at both ends of tube were removed, fixed, sectioned, and immunostained.

RESULTS
Histopathological Findings

Altogether, 21 VS tumors from NF2 cases and 23 sporadic VS cases were studied. NF2 patients tended to be younger, with larger tumors than were found in the unilateral cases (Table 2). These results correlate with the clinical course, showing that bilateral tumors have faster growth rates than do unilateral tumors (Schuknecht, 1993). All 44 tumors had similar histological features. Morphologically, the tumors were composed of merging and diverging streams of elongated spindle cells with long cigar-like nuclei (Linthicum and Brackmann, 1980). All tumor cells had lost the one-to-

TABLE 2. *Average Age at Surgery, Vestibular Schwannoma Size, Number of Cases With a Surface or Infiltrating Nerve, and MAG Expression by NF2 and Sporadic Vestibular Schwannomas*

	Avg age (yr)	Avg tumor size (cm)	Surface nerve (no. of cases)	Infiltrating nerve (no. of cases)	MAG expression (no. of cases)
NF2 (n = 21)	24.6	3.3	5	13	18
Non-NF2 (n = 23)	48.2	1.8	10	2	11

one relationship with the nerve axon. In some areas, the cells were arranged in whorls (grape-like lobules) (Louis et al., 1995). The cells were arranged in fascicles and loops, and palisading of the cell nuclei could be observed. We observed that the bilateral NF2 tumors had more grape-like lobule structures and a significant number of fine residual nerve fibers inside the tumor relative to unilateral tumors (Fig. 1a). The unilateral sporadic tumors contained more nerves on the surface, rather than the great amount of infiltrating nerve fibers found in NF2 tumors (Fig. 1b and Table 2).

Normal Myelin Proteins Are Expressed in Residual Nerve, But Not in Tumor Cells

Immunohistochemistry and Western blot were used to detect the expression of myelin proteins in the denervated schwannoma cells. Expression of several major myelin sheath proteins (P0, PMP22, and MBP) and myelin glycosphingolipids (GalC) was generally not seen in the tumor cells by immunostaining. Five cases had low levels of P0 protein in focal areas of tumor cells (see T3 and T4 in Fig. 2f). However, the myelin sheath proteins were expressed in the compressed nerve tissues and in the fine nerve fibers (Fig. 1a). The results of downregulation of these compact myelin proteins in schwannoma cells may be attributable to loss of axonal contact (LeBlanc and Poduslo, 1990). Interestingly, the expression of MAG can be detected in most schwannoma tissues (Table 2). Immunohistochemical staining of the tumors showed that, compared with MAG levels in the myelin sheath of normal nerves, the tumors displayed reduced expression of MAG (Fig. 1c). Furthermore, in normal compressed nerve fibers, strong MAG staining was localized to the periaxonal region of the Schwann cells, whereas light staining could be seen in the compact myelin portion. A similar observation has been previously reported, but it was suggested that the positive MAG staining in the compact myelin was due to a staining artifact (Trapp and Quarles, 1984). Yet, we observed no nonspecific background staining with frozen tissue sections. To substantiate the immunostaining observations, a single 100-kD protein corresponding to MAG was detected (Fig. 2a). The pattern of MAG expression appeared to be heterogeneous and varied from one tumor to another (Fig. 2a), even within cells of the same tumor. Interestingly, 18 of 21 (86%) NF2 tumors expressed MAG relative to 11 of 23 (48%) unilateral tumors ($P = 0.08$). Low levels of MAG expression were seen in regions where the cells were arranged in palisading structures (Fig. 1d). MAG ex-
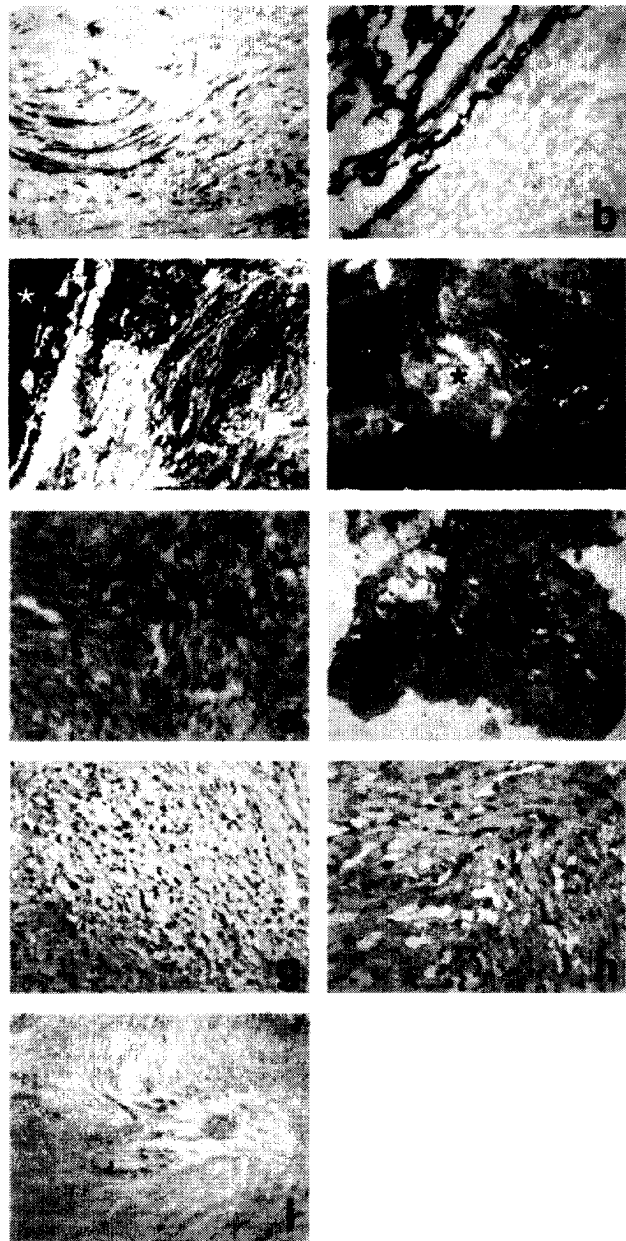

Fig. 1. Immunohistochemical staining of human vestibular nerve tissues. Frozen sections were stained with antibody against protein zero (P0) [the residual nerve fiber in a bilateral tumor (a) and a unilateral tumor (b)]; myelin-associated glycoprotein (MAG) [MAG expression in the tumor cells (T) and in the residual nerve (white star) (c), lower MAG expression in tumor cells with palisading structure (black star) (d)]; neural cell adhesion molecule (N-CAM) (e); L1 (f); SCIP (g); Krox-20 (h); Krox-24 (i). a: ×200; b–i: × 400.
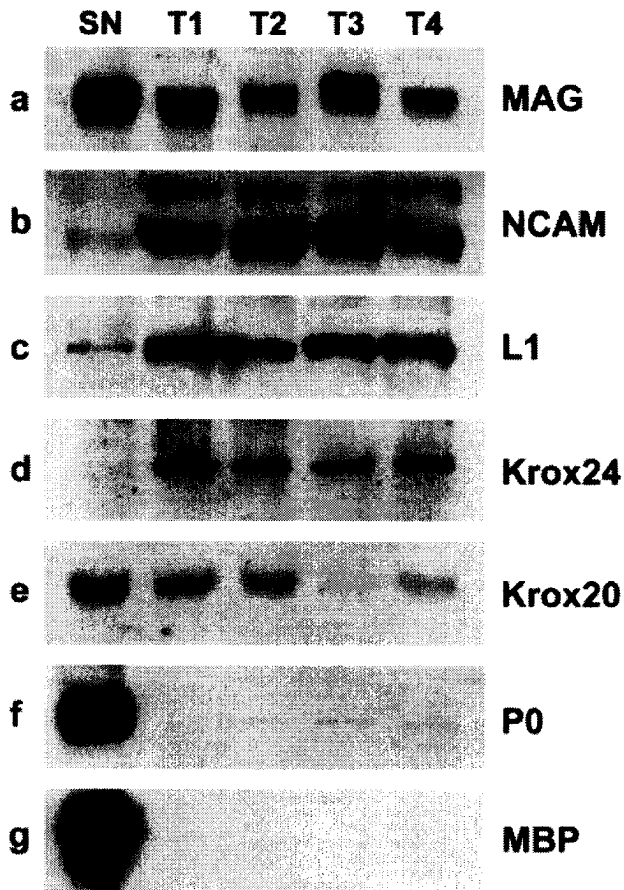
Fig. 2. Protein lysates (25 µg) from four different human vestibular schwannoma tumor samples (T1–T4) and one normal sciatic nerve tissue (SN) were separated by 8% SDS-PAGE and immunoblotted with antibody of (a) myelin-associated glycoprotein (MAG), (b) neural cell adhesion molecule (N-CAM), (c) L1, (d) Krox24, (e) Krox20, (f) P0, and (g) myelin basic protein (MBP).

pression levels have been correlated with the density of Schwann cells in culture (Sasagasako et al., 1996). Using Ki67, a marker for cell proliferation, we searched for an association between MAG expression and cell proliferation, but no such correlation was observed (data not shown).

Upregulation of the Neural Adhesion Molecule N-CAM, L1, and NGFR Expression in Schwannoma Cells

Using both Western blot and immunohistochemistry, we were able to detect homogeneous expression of N-CAM and L1 in the tumor cells (Fig. 1e,f). A similar pattern was also seen for NGFR expression in all tumors (data not shown). Three size variants of N-CAM (125 kD, 145 kD, and 180 kD) and the 200-kD L1 were detectable in Western blots of tumor lysates (Fig. 2b,c). In contrast, N-CAM and L1 were detectable in less than 10% of the cells in normal nerves, which are

presumably nonmyelinating Schwann cells (data not shown). Upregulation of NGFR has been reported in schwannoma cases (Hoshi et al, 1994), and increased expression of NGFR has been correlated with upregulation of Krox24 (Nikam et al., 1995). These results suggest that the schwannoma cells were not in a myelinating stage.

SCIP Is Expressed in Both Normal and Tumor Cells

SCIP is transiently expressed in pro-myelinating Schwann cells (Zorick et al., 1996). In an effort to clarify the lineage of schwannoma cells further, we examined SCIP expression in primary VS cells. A high-affinity SCIP-specific monoclonal antibody (Zorick et al., 1996) was used to detect SCIP expression in situ. A heterogeneous expression pattern (10–95% staining of cells in a section) was seen in both Schwann cells and schwannoma cells (Fig. 1g). In most cases, the schwannoma cells had a stronger staining density when compared with Schwann cells. Less than 5% of Schwann cells were positive for SCIP expression found in the residual nerve tissues.

Both Krox20 and Krox24 Are Expressed in Tumor Cells

In Schwann cells, Krox20 and Krox24 appear to have antagonistic affects. Krox24 is expressed in most Schwann cell precursors at birth, before the onset of myelination, whereas only 30% of these cells express Krox20 (Topilko et al., 1997). In adult animals, almost all myelinating Schwann cells express Krox20, whereas few cells express Krox24. Antagonistic regulation of Krox20 and Krox24 has also been noted in Wallerian degeneration and peripheral neuropathies (Topilko et al., 1997). In human vestibular schwannomas, we were able to detect the expression of both proteins in vestibular schwannoma cells. A 50-kD band corresponding to Krox20 and a 67-kD band corresponding to Krox24 were seen on Western blots of tumor lysates (Fig. 2d,e). In tumor sections, both proteins were detected in the nuclei, which exhibited a heterogeneous expression pattern (Fig. 1h,i). No notable difference between bilateral and unilateral tumors for Krox20 expression was seen, as well as an average 44% of Krox20 expression cells in both types of tumor. In contrast, Krox24 is expressed in 25% of cells in NF2 tumors and 14% of cells in unilateral tumors. In two NF2 tumors, almost negative staining was seen for both proteins. However, more than 95% of the cells expressed SCIP (data not shown), confirming the previous observation in Krox20$^{-/-}$ mice with upregulation of SCIP (Zorick et al., 1999). In the residual normal vestibular nerves, more than 50% of the Schwann cells expressed Krox20, whereas less than 5% stained for Krox24.
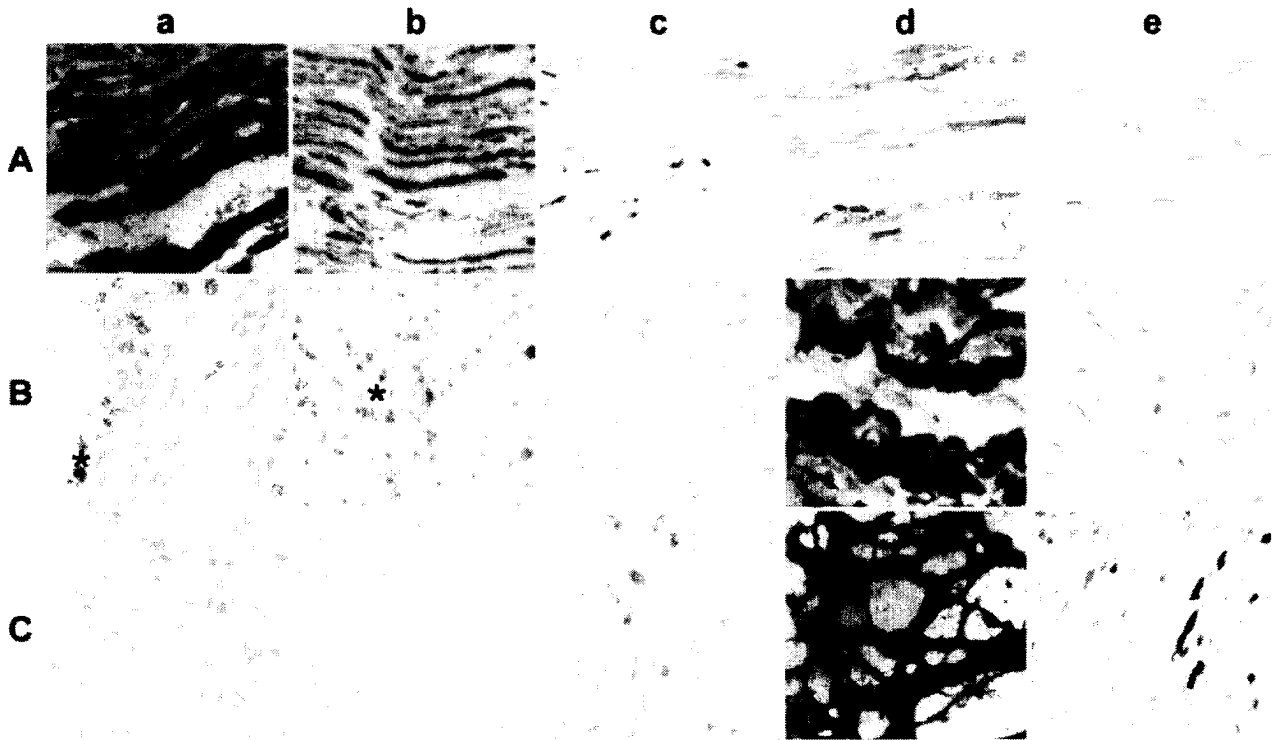
Fig. 3. Immunohistochemistry staining on normal human nerve (A), transplanted human nerve tissues (B), and schwannoma (C). The frozen sections were stained for protein zero (P0) (a), PMP22 (b), Krox20 (c), nerve growth factor receptor (NGFR) (d), and Ki67 (e).

Except for residual degraded myelin proteins (black star) (P0, PMP22) and negative for Ki67, the transplanted nerves have gene expression similar to that of schwannoma.

Wallerian Degeneration Model

To further confirm the similarity of genetic profile of schwannoma cell and dedifferentiated Schwann cells during Wallerian degeneration, the fresh isolated human sciatic nerve tissue were transplanted subcutaneously into nude rats. The protein expression of P0, PMP22, Krox20, NGFR, and proliferating cell nuclear antigen (PCNA) was examined by immunostaining. The expression profiles of degenerated Schwann cells and schwannoma cells were similar. However, higher proliferation rates in schwannoma tumors were noted (Fig. 3).

Interaction of Schwannoma and Regenerating Axons

During nerve injury, the demyelinated Schwann cells in the distal portion of the nerve are able to redifferentiate, when nerve regeneration occurs. Using
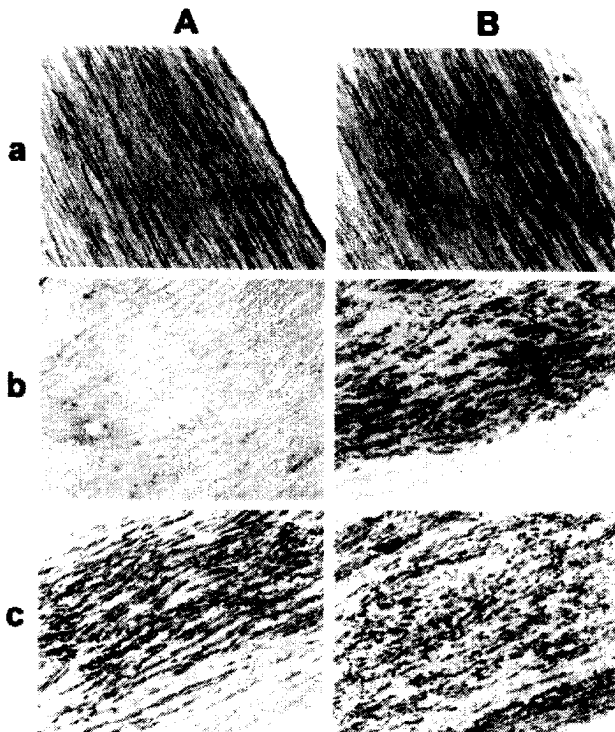

Fig. 4. Tissue sections of transplanted human schwannoma cells (A) and normal human Schwann cell control (B) were stained with neurofilament (a), a human protein zero (P0)-specific antibody (b) and an antibody recognizing both human and rat P0 (c) ×400. The transplanted human schwannoma cells failed to stain positively for human P0 protein.

an in vivo transplantation model, our goal was to examine whether the NF2 gene mutated schwannoma cells are able to redifferentiate and remyelinate the axon. Primary human vestibular schwannoma cultures from three different patients, and from a control subject, of normal human Schwann cell cultures were implanted, respectively. At 4 weeks post-implantation, both normal Schwann cells and schwannoma cell-filled tubes were removed and examined for nerve regeneration and myelination. We found that the transected proximal end of the sciatic nerve was able to regenerate into the implanted human schwannoma cells by staining with neurofilament (Fig. 4). A human P0-specific antibody was then used to determine whether the nerves were myelinated by either rat or human Schwann cells (Bollensen et al., 1990). We confirmed the finding reported by Levi and Bunge (1994) that ex vivo expanded normal human Schwann cells can be regulated and redifferentiated into myelinating Schwann cells to myelinate the rat regenerating nerve fibers efficiently (Fig. 4). However, the human schwannoma cells failed to cover the nerve with human myelin. These data suggest that the transformed schwannoma cells, which have lost NF2 gene function, cannot be induced to redifferentiate as normal Schwann cells during nerve regeneration.

DISCUSSION

Our data suggest that loss of NF2 gene function in Schwann cells may lead to their dedifferentiation from a myelinating stage to a nonmyelinating neoplastic stage. Human vestibular schwannoma cells have downregulated myelin proteins (P0, PMP22, and MBP) and galactocerebroside (GalC) and have upregulated N-CAM, L1, NGFR, and expressing transcription factors SCIP, Krox20, and Krox24. Taken together, the expression profile of the vestibular schwannomas most closely resembles that of demyelinated Schwann cells during Wallerian degeneration. However, in Wallerian degeneration, the demyelinated Schwann cells are non-neoplastic. Schwann cell dedifferentiation is caused by loss of axonal contact; the cells are able to redifferentiate to remyelinate the regenerating axon. The schwannoma cells are transformed with a high proliferation rate; the cell dedifferentiation is caused by NF2 mutation. These dedifferentiated schwannoma cells are unable to remyelinate the axons. Our data in this study suggest that the NF2 gene may have a key role in the transition from the promyelination stage to the myelination stage, the period during which NF2 is highly expressed (Scherer and Gutmann, 1996). Whether the loss of NF2 function will downregulate myelin gene expression directly in Schwann cells remains speculative. If the NF2 gene not only plays a role in Schwann cell differentiation but is also involved in the myelination process, it might explain why the Schwann cell is the primary target for the NF2.

To our surprise, expression of MAG, a noncompact myelin component, is seen in most tumors. MAG is thought to be regulated by axonal contact, and its expression is thought to be required for P0 and MBP expression (LeBlanc and Poduslo, 1990). MAG expression depends on contact between the axon and Schwann cells and is required for P0 and MBP expression (LeBlanc and Poduslo, 1990). Like P0, MAG is also expressed in immortalized rat Schwann cells lines (Goda et al., 1991; Toda et al., 1994), and the level of expression of MAG in these cells appears to be associated with cell–cell contact (Sasagasako et al., 1996). It appears that transformation of Schwann cells, either by immortalization or during tumor formation, enabled the Schwann cell to express MAG without axonal contact.

In this study, we also demonstrate that different levels of MAG expression are associated with different histological patterns. It remains unclear, however, why levels of MAG expression are lower in cells arranged in palisading structures. Staining of the tumors with the cell proliferation marker Ki67 indicate that there was no correlation between MAG expression and the rate of cell division.

The denervated schwannoma cells also expressed all three transcription factors (Krox20, Krox24, and SCIP), which were axonally regulated (Murphy, 1996; Scherer, 1994; Zorick et al., 1996). During nerve regeneration, SCIP and Krox20 are reexpressed by the Schwann cells after establishing contact with the regenerated axon (Zorick et al., 1996). The independent of axonal regulation of SCIP, Krox20, and Krox24 expression in schwannoma cells may exhibit a neoplastic phenotype.

Krox24 and Krox20, also known as egr-1 and egr-2, respectively, were originally identified as growth response genes; more recent evidence suggests they operate as the tumor suppressor genes (Topilko et al., 1997; Lanoix et al., 1998). Except for two NF2 tumors, Krox20 and Krox24 were detected the schwannomas suggesting either a feedback mechanism, or alternatively, they may be the result of a defective regulatory pathway.

In summary, the results of this study suggest that neoplastic schwannoma cells have similar characteristics to those exhibited by Schwann cells in the promyelination stage. Schwannoma cells at this stage have no detectable P0, MBP, or PMP22, compact myelin protein GAP43, and GalC. Schwannoma cells do express N-CAM, L-1, MAG, Krox20, Krox24, and SCIP. In addition, we hypothesize that the loss of NF2 gene function causes Schwann cells to dedifferentiated from quiescent, myelinating cells into proliferating, pro-myelinating tumor cells.

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